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(54) Title: METHOD OF TARGETING DNA**(57) Abstract**

The present invention relates to a method of forming a three-stranded DNA molecule wherein each strand of the three-stranded DNA molecule is hybridized (that is, non-covalently bound) to at least one other strand of the three-stranded DNA molecule. The method comprises: contacting a recombination protein with a double-stranded DNA molecule and with a single-stranded DNA molecule sufficiently complementary to one strand of the double-stranded DNA molecule to hybridize therewith, which contacting is effected under conditions such that the single-stranded DNA molecule hybridizes to the double-stranded molecule so that the three-stranded DNA molecule is formed.

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METHOD OF TARGETING DNA

This is a continuation-in-part of Application No. 07/611,268, filed November 9, 1990, the entire contents of which are incorporated herein by reference.

BACKGROUND ON THE INVENTION

Technical Field

The present invention relates, in general, to a method of forming triplex DNA and to a method of achieving sequence specific cleavage of duplex DNA using such a triplex. The present invention further relates to a method of targeting DNA and, in particular, to a method of effecting sequence-specific targeting of DNA.

15 Background Information

Several groups have recently reported highly efficient cleavage of genomic DNA at specific sequences. For example, Szybalski and coworkers have cleaved Saccharomyces cerevisiae and E. coli genomes at a single introduced lac operator site (Koob et al, *Science* 250, 271 (1990)). These investigators first methylated Hae II sites in the DNA while using the lac repressor to protect the lac operator from methylation. After inactivation of the methylase and the repressor, the only Hae II site unmodified and available for cleavage was the lac operator site. The advantages of this approach were the high yield and high specificity. The disadvantage was that only a lac operator site could be cleaved.

30 A second approach was used by several investigators to cleave genomes as large as S. cerevisiae. This approach used the ability of synthetic homopyrimidine

ligonucleotides to anneal to duplex homopyrimidine-homopurine tracts to form triple-helical structures. This approach was first used by Moser and Dervan (*Science* 238, 645 (1987)) to cleave a plasmid by equipping the oligonucleotide with an EDTA-Fe cleavage moiety. Subsequently, other cleavage moieties were attached to homopyrimidine oligonucleotides. For example, Schultz and coworkers attached staphylococcal nuclease (Pei et al, *Proc. Natl. Acad. Sci. USA* 87, 9858 (1990)), and Helene and coworkers attached a phenanthroline-copper derivative (Francois et al, *Proc. Natl. Acad. Sci. USA* 86, 9702 (1989)) as cleavage moieties. Dervan and coworkers have also used a guanine-rich cleavage oligonucleotide to form a triplex (Beal et al, *Science* 251, 1360 (1991)), and have cleaved DNA using a triplex and the methylation protection strategy described above (Strobel et al, *Nature* 350, 172 (1991)). The advantages of this targeting approach are efficiency and the ability to use oligonucleotides with a variety of derivatives. The disadvantage is that only homopyrimidine or guanine-rich oligonucleotides have been used successfully.

The practical use of previously reported strategies is severely limited because of the paucity, or indeed the complete absence, of possible cleavage sites in any particular DNA sequence. The present invention, on the other hand, provides a general method of cleaving DNA at any desired site, or pair of sites. Site-specific cleavage, however, is only a single embodiment of the present invention. In a broader sense, the invention relates to a method of targeting any desired sequence

specifically and efficiently whether, it be for purposes of cleavage, protection or enrichment.

SUMMARY OF THE INVENTION

It is a general object of the invention to provide
5 a method of forming triplex DNA.

It is another object of the invention to provide a method of identifying the presence of a specific DNA sequence in a DNA-containing sample.

It is another object of the invention to provide a
10 method of inhibiting transcription of specific gene sequences.

It is an object of the invention to provide a rapid, efficient and general method of effecting sequence-specific targeting of DNA for purposes of, for
15 example, cleavage, protection or enrichment.

The present invention relates to a method of forming a three-stranded DNA molecule wherein each strand of the three-stranded DNA molecule is hybridized (that is, non-covalently bound) to at least one other
20 strand of the three-stranded DNA molecule. The method comprises:

contacting a recombination protein with a double-stranded DNA molecule and with a single-stranded DNA molecule sufficiently complementary to one strand of
25 the double-stranded DNA molecule to hybridize therewith, which contacting is effected under conditions such that the single-stranded DNA molecule hybridizes to the double-stranded molecule so that the three stranded DNA molecule is formed.

30 Further objects, and advantages, will become clear from a reading of the disclosure that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1 shows two possible joint molecule structures formed by recombinase protein between a linear duplex and a homologous single-strand circular DNA. Top, a joint molecule having heteroduplex DNA and a displaced strand. The presence of a displaced strand allows branch migration to occur resulting in rapid dissociation of the joint substrates when heteroduplex regions are short. Bottom, a stable joint molecule in which the three strands are associated by additional noncovalent interactions and cannot participate in branch migration. The arrangement of the three strands is only schematic, and no disruption of the starting duplex is implied.
- Figure 2 shows that recombinases form joint molecules using short homologies. A. partial map of the pGem 4 linear duplex substrate used in joint molecule assays is shown. Boxes indicate regions homologous to M13mp18. Numbers in the polylinker region at the right of the duplex indicate the lengths of homology available for pairing with M13mp18 single-strand DNA when pGem 4 is linearized at each of these sites. B - D. Joint molecule assays using pGem 4 linear duplex and M13mp18 viral strand DNA and HeLa recombinase fraction (panel B), *Drosophila* recombinase fraction (panel C) or *E.coli* recA and SSB proteins (panel D) were carried out as described in the Examples and were deproteinized by the addition of SDS. Lengths of homology available for pairing are indicated. Lane C, control, DNAs incubated alone; lane 1, Hind III-linearized pGem 4; lane 2, Sal I-linearized duplex; lane 3, Bam HI-linearized duplex; lane 4, Kpn I-linearized duplex; lane 5, Eco RI-linearized duplex; lane 6, Duplexes digested with both Hind III and Eco RI

(panels B and D), or ligation contr 1, duplex alone (panel C).

Figure 3 demonstrates novel joint molecule assay.

A. Scheme for joint molecule formation between a linear duplex DNA and a homologous ³²P-labeled oligonucleotide. B. Joint molecule assays were performed at 37°C for 10 min with HeLa recombinase fraction, 100 ng of Hind III-linearized pdel 9 duplex and 5 ng of a homologous ³²P-labeled oligonucleotide 33 bases long (lane 1), 20 bases long (lane 3) or 13 bases long (lane 5). Samples were deproteinized with 1% SDS. Control experiments in lanes 2, 4 and 6 demonstrate that joint molecules are not formed by potential contaminating exonucleases. Duplexes and oligonucleotides (33-mer, lane 2, 20-mer, lane 4 and 13-mer, lane 6) were separately incubated with recombinase at 37°C, brought to 1% SDS and then combined and annealed at 65°C without quenching on ice prior to electrophoresis at room temperature. Under these annealing conditions, DNA duplexes 33 or 20 bp long can be formed and are stable.

Figure 4 depicts the thermal stability of joint molecules. A. Scheme for determining relative thermal stabilities of short duplex regions, I, branch migration structures, II, or deproteinized joint molecules formed by recombinase, III. B. Representative data from thermal stability assays for short duplexes, branch migration structures and joint molecules involving a region of shared homology 38 base pairs long. Joint molecules were formed by HeLa recombinase fraction between M13mp18 single-strand DNA and Sal I-linearized pGem 4 duplex DNA and deproteinized by the addition of SDS. Stability of both the duplex and the branch migration structure was

monitored after agarose gel electrophoresis as loss of ^{32}P -label migrating at the position of M13mp19 single-strand DNA.

Figure 5 summarizes the thermal stability data.

- 5 Thermal stability assays were carried out as described in the Examples for short duplexes, branch migration structures and joint molecules having 13, 26, 38 or 56 bp of homology. The indicated temperatures are those which resulted in dissociation of greater than 50% of the structures after a 10 min incubation. Because of the instability of the 13 bp duplex at 37°C, the stability of the corresponding 13 bp branch migration structure was not determined.

- Figure 6 shows stable joint molecules having three intact DNA strands. A. A joint molecule formed from a Hind III-Bgl I fragment of pGem 4 having a unique ^{32}P -label at the 3' end of the plus strand and sharing 56 bp of homology with M13mp18. B. Thermal stability assay of ^{32}P -labeled joint molecules formed by recA protein. C. Comparison of the relative thermal stabilities of ^{32}P -labeled joint molecules formed by E. coli recA protein, filled circles, HeLa recombinase fraction, open circles, and the corresponding 56 bp branch migration structure, triangles.

- 25 Figure 7 depicts stable joint molecules in the absence of recA protein. A. Joint molecules formed by recA were deproteinized as described in the Examples and analyzed for residual recA protein on a 12% SDS polyacrylamide gel followed by Western blotting with anti-recA antibody and ^{125}I -labeled secondary antibody. Lanes 1-5, 5 ng, 1 ng, 0.5 ng, 0.2 ng, and 0.1 ng, respectively, of purified E.coli recA protein; lane 6, total deproteinized joint molecules from five assays; lane 7, 0.2 ng purified recA protein. B. Thermal

stability assays of the deproteinized joint molecules. Lane 1, DNA substrates alone; lane 2, a reacted joint molecule assay with recA stopped with SDS and EDTA and loaded on the agarose gel directly; lanes 3-7, thermal stability assays of deproteinized joint molecules carried out as described in Figure 4. ds and ss indicate mobilities of double-strand and single-strand DNA, respectively.

Figure 8 proposes a pairing scheme for a triple helix formed by recombinase proteins. A. Pairing of a third strand to a homologous duplex in the major groove. Duplex strands retain Watson-Crick base pairing shown by solid lines. Non-Watson-Crick pairing involving the third strand and a purine in either the Watson (W) or Crick (C) strand of the duplex is shown by the dotted line. Shaded area represents the phosphate backbone of each strand. Note that the third strand is parallel to the identical Watson strand. B. Proposed hydrogen bonding scheme for all four possible base triplets. Cytosine residues in the third strand are protonated at the N₃ position to allow formation of two hydrogen bonds.

FIGURE 9. Scheme for the formation of synaptic complexes and stable joint molecules. A duplex DNA and a homologous oligonucleotide are incubated in the presence of *E. coli* recA protein to form a synaptic complex in which the two DNAs are paired within a recA nucleoprotein filament. The formation of synaptic complexes is monitored by the inability of a restriction endonuclease to cleave the duplex within the region of pairing. If recA protein is removed from synaptic complexes by the addition of SDS detergent, stable, deproteinized joint molecules result.

FIGURE 10. Synaptic complexes and joint molecules formed by recA. Fig. 10A. Oligonucleotides having 56, 38, 26 or 20 bases of homology to pUC18 duplex DNA that span a Sac I site. Fig. 10B. Synaptic complexes formed by recA. ³²P-labeled oligonucleotides, pUC 18 supercoiled plasmid DNA and recA protein were co-incubated. Following incubation with the appropriate restriction endonuclease, the reactions were brought to 1% SDS and electrophoresed on a 1% agarose gel containing ethidium bromide. The footprint of synaptic complexes is represented by the presence of supercoiled plasmid DNA remaining after incubation with a restriction endonuclease. Fig. 10C. Joint molecules formed by recA. An autoradiogram of the same agarose gel demonstrates the formation of joint molecules as indicated by the presence of ³²P-label migrating at the position of supercoiled plasmid DNA. 1, linear duplex; sc, supercoiled duplex.

FIGURE 11. Formation of synaptic complexes with linear duplexes. Fig. 11A. Map of the DNA substrates showing the 33-base oligonucleotide homologous to the linear pBR322 duplex and the location of the Cla I site. Fig. 11B. Synaptic complex assay with a linear duplex. The nonhomologous oligonucleotide is an M13mp18 sequence corresponding to positions 6228-6260. H, homologous 33-base oligonucleotide; NH, nonhomologous 33-base oligonucleotide.

FIGURE 12. Extent of the restriction endonuclease footprint of synaptic complexes. Synaptic complexes were formed with a homologous 20-base oligonucleotide and pUC18 duplex DNA in the presence of recA. The complexes were incubated with a variety of restriction endonucleases whose cleavage sites are indicated by the arrows. Protection from cleavage afforded by the

synaptic complex extended to Sac I and Sph I sites. No protection was observed at Eco RI or Hind III sites. Numbers indicate the length from the end of the oligonucleotide to the proximal cleavage site.

5 FIGURE 13. The effect of directionality on synaptic complex and joint molecule formation. Fig. 13A. A 56-base oligonucleotide completely homologous to the polylinker region of pUC18 or having additional nonhomologous sequences at either or both the 5' and 3' ends. Fig. 13B. Formation of synaptic complexes by 10 recA can initiate at either the 5' or 3' end of the single-strand or at an internal site. Synaptic complex assays were carried out with ³²P-labeled oligonucleotides. The band in lane 1 migrating just 15 above linear pUC18 represents nicked duplex present in the starting substrate. Fig. 13C. Homology at the 5' end of the single-strand is preferred in joint molecule formation. Synaptic complex assays were deproteinized by the addition of SDS followed by electrophoresis and 20 autoradiography. Lanes 1-8 correspond to lanes 4-11 in part B above. 1, linear duplex; sc, supercoiled duplex.

FIGURE 14. The minimal searching unit for homologous pairings. Fig. 14A. Oligonucleotides 33, 25 15 or 13 bases long are homologous to pBR322. Positions of Eco RI(E), Cla I (C) and Hind III (H) restriction endonuclease sites in the duplex are shown. Fig. 14B. Nucleotide sequence of the 15-base oligonucleotide and corresponding duplex sequence 30 showing positions of Cla I and Hind III cleavage. Bases in the duplex that comprise all or part of the recognition sequence for Cla I and Hind III are indicated in bold. Fig. 14C. Formation of synaptic complexes with an oligonucleotide 15 bases long.

Synaptic complexes were formed with the 15-base oligonucleotide as described above. Lanes 2-4, incubation with Hind III; lanes 5-7, incubation with Cla I; lanes 8-10, incubation with Eco RI.

5 FIGURE 15. RecA pairs less than one helical repeat of the duplex DNA. Fig. 15A. Formation of synaptic complexes with the L series of oligonucleotides. Results represent the average of three independent observations. Percent protection of the duplex is
10 normalized to a control reaction containing duplex DNA and recA. Error bars represent the standard error of the mean. Fig. 15B. Formation of synaptic complexes with the R series (solid line). Results represent the average of four independent observations. Shown for
15 comparison is the corresponding data for the L series, dotted line. The detection and quantitation of small numbers of synaptic complexes was facilitated by the use of 200 ng of duplex DNA in these experiments.

FIGURE 16. The specificity of the recA pairing
20 reaction. Fig. 16A. A 30-base oligonucleotide homologous to M13mp18 spans one of three NdeI restriction endonuclease sites (N) in the duplex. The oligonucleotide sequence is
5'TATCAACCGGGTACATATGATTGACATGC 3'. The NdeI site is
25 in bold. Fig. 16B. RecA targets synaptic complex formation to the homologous site in the duplex with high efficiency. The 30-base oligonucleotide was incubated with duplex DNA and recA in a synaptic complex assay followed by incubation with NdeI. Size
30 markers (M) are lambda Hind III and phi X 174 Hae III fragments. Lane 6, M13mp18 duplex DNA digested with Bam HI yielding a full-length 7.2 kb linear fragment. For clarity, the ethidium-bromide stained gel is reproduced in reverse contrast.

FIGURE 17. Schematic of the strategy used for sequence specific cleavage of DNA. This diagram shows cleavage at a single site.

FIGURE 18. Fig. 18A. A schematic showing the position of cleavage of lambda DNA using an oligonucleotide homologous to the site shown by the bold arrow. Lambda DNA contains 5 Eco RI sites, including the one shown by the bold arrow. Fig. 18B. Agarose gel stained with ethidium bromide showing sequence-specific cleavage of lambda DNA. Lane 2 shows the complete cleavage reaction and the other lanes had components omitted as shown. Unmethylated lambda DNA was first protected by incubating with recA protein and an oligonucleotide 30 bases long identical to the lambda sequence from position 31,734 to 31,763. The sequence was 5'-TCACGCCGGAAGTGAATTCAAACAGGGTTC-3'. After 10 minutes at 37°C, a minimal volume of Eco RI methylase and S-adenosylmethionine was added and the reaction was allowed to proceed for 20 minutes. The recA protein and methylase were then inactivated by heating for 15 minutes at 65°C. Eco RI restriction enzyme was added to the tube at 37°C, and the reaction was allowed to proceed for 60 minutes. The reaction volume was 40 µl and contained, in order of addition: 25 mM Tris-acetate (pH 7.5), 4 mM Mg-acetate, 0.4 mM dithiothreitol, 0.5 mM spermidine, 10 µg of recA protein, 100 µM EGTA, 1.1 mM ADP, 0.3 mM ATP-gamma-S (Fluka BioChemica), 0.18 µg of oligonucleotide, 0.9 µg of lambda DNA, 4 µg of acetylated bovine serum albumin (BSA), 3.8 units of Eco RI methylase, 120 µM S-adenosylmethionine, and 20 units of Eco RI restriction enzyme (all reagents listed from lambda DNA on were from New England Biolabs). The Tris-acetate, dithiothreitol, spermidine, and buffers used in the

final recA protein purification steps were passed through Chelex 100 (Bio-Rad) columns to remove trace metal contaminants. The reactions were stopped with 5 μ l of 6% sodium dodecyl sulfate (SDS), 90 mM EDTA and 0.1% bromophenol blue. 20 μ l of the final reaction mixtures were mixed with 60 μ l of 0.5% InCert agarose at 65°C and allowed to set in the wells of a 1.5% agarose gel. The gel was run by pulsed field electrophoresis on a CHIEF-DR11 system (Bio-Rad) for 36 hours at 12°C, 180 V, and 2.5 s switch time.

FIGURE 19. Fig. 19A. A schematic showing the positions of cleavage of the *E. coli* chromosome using two oligonucleotides homologous to sites in the *uvrB* and *topA* genes. Fig. 19B. Agarose gel stained with ethidium bromide showing sequence-specific cleavage of *E. coli* DNA generating a 520 kb fragment. The compression (C) zone of the gel is also shown. Lane Y, yeast *S. cerevisiae* chromosomal DNA markers. Lane lambda, lambda concatamer DNA ladder. Lane E, unmodified *E. coli* DNA after a complete digestion by Eco RI. Lanes 1-5, complete cleavage reactions with different amounts of oligonucleotide in each lane. The *uvrB* oligonucleotide sequence was 5'-TCATGAGTAAACCGTTCAAACCTGAATTCGCTTTTA-3' (36 bases long), and the *topA* sequence was 5'-CGAGATCGAAGAGGGCGAATTCGCGATTAA-3' (30 bases long). Reaction conditions were similar to those of Figure 18, except the following conditions were modified to obtain good results for agarose-embedded DNA. RecA protein and oligonucleotide were preincubated with the DNA for 15 minutes at 37°C; methylase and S-adenosylmethionine were added and the methylation was allowed to proceed for 1 hour. The methylation was terminated by adding 100 μ l of 2% SDS for 30 minutes at 37°C. The beads

were then equilibrated in 100 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1.5 μ M dithiothreitol, and 200 μ g/ml nonacetylated BSA (Calbiochem-Behring). The observation of Wilson and Hoffman (Wilson et al, *Anal. Biochem.* 191, 370 (1990)), that this buffer is excellent for inhibiting nonspecific or star activity of Eco RI on agarose-embedded DNA, was confirmed. Concentrations of other reagents are as in Figure 18 except that each tube contained 20 μ g recA protein, the indicated amount of each oligonucleotide, 30 μ l (packed volume) of beads containing *E. coli* DNA, 40 units of methylase, and digestion was with 40 units of Eco RI restriction enzyme. After stopping the reaction, the beads were run on a 1% agarose gel for 30 hours at 12°C, 160 V, with the switch time ranged from 60-140 s. Fig. 19C. Southern blot of the gel in part (B). The gel was blotted onto a GeneScreen Plus nylon membrane (Dupont) according to the manufacturer's directions. The probe was made by polymerase-chain-reaction amplification (PCR) of a 600 base pair fragment of the *trpA* gene from *E. coli* using 32 P-deoxycytidine 5'-triphosphate. The *trpA* gene lies between the *uvrB* and the *topA* gene. The film was overexposed to reveal minor bands, but densitometry was performed on less exposed films. Lane E, which contained the same amount of DNA as lanes 1-5, showed the hybridization of the probe to the predicted 40 kb fragment generated by complete Eco RI digestion (Kohara et al, *Cell* 50, 495 (1987)); the intensity of this band provided the 100% value to calculate the 520 kb fragment yield.

FIGURE 20. Fig. 20A. A schematic showing the positions of cleavage of the human CF locus using two oligonucleotides homologous to sites in intron 1 and exon 19. The gene contains a total of 24 exons. Fig.

20B. Agarose gel stained with ethidium bromide showing development of smaller fragments of DNA as the oligonucleotide concentration was decreased. Lane S, Sfi I digest of unmodified HeLa cell DNA. Lane lambda, lambda concatamer DNA ladder. Lanes 1-6, complete cleavage reactions with the indicated amount of each oligonucleotide. The intron 1 oligonucleotide sequence was 5'-TAAGTGCTCAGAAAACATTTCTTGACTGAATTCAGCCAACAA-AAATTTTGGGGTAGGTAG-3' (60 bases long), and the exon 19 oligonucleotide sequence was 5'-AATGGCCAACTCTCGAAAGTTATGATTATTGAGAATTCACACGTGAAGAAAGATGACATCTGG-3' (63 bases long). Conditions were identical to Figure 19 except that the reaction volume at all steps was doubled, and 25 μ l (packed volume) of HeLa beads were used per reaction. 80 units of Sfi I (New England Biolabs) were used in lane S according to the manufacturer's directions. A 1% agarose gel was run for 32 hours at 12°C, 160 V, with the switch time ramped from 40-120 s. Fig. 20C. Southern blot of the gel in part (B). The Sfi I digest band was 270 kb long and was used in calculating the yield of the 180 kb fragment. The probe was made by PCR of the CF cDNA T8-B3 plasmid (from the American Type Culture Collection). The probe was 550 bases long and contained 410 bases of exon 13 colinear with 140 bases of exon 14.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates, at least in part, to a three-stranded DNA molecule in which non-covalent interactions are formed between a DNA duplex and a third DNA strand. The invention further relates to an enzymatic method of forming such a triplex.

The triplex of the present invention can be formed by contacting a recombinase protein with a DNA duplex and a single stranded DNA sequence under conditions

such that hybridization between the duplex and the single stranded molecule is effected. Recombinase proteins include proteins that carry out *in vitro* biochemical steps that mimic homologous recombination in cells or *in vivo* and, in particular, that can pair two DNAs, single-stranded or double-stranded, in a homology-dependent manner. Examples of such proteins include procaryotic proteins such as *E. coli* recA protein and other bacterial recA analogues, and the bacteriophage T4 uvsX protein. Proteins from eukaryotic sources have also been described and are referenced above.

The triplex of the invention is stable even upon removal of the recombinase protein. The complementary strands of the duplex can have any base sequence and the single-stranded molecule can be complementary to as few as 13 bases of the duplex strands for a stable triplex to form. One skilled in the art will appreciate that the minimum number of base pairs of homology required may vary with the recombinase protein used. For example, as few as about 13 bp of homology is recognized by human and *Drosophila* recombinase, and 38 bp by *E. coli* recA.

Recombinase protein suitable for use in the present method can be present in purified form or can be partially purified. As noted above, the enzyme can be isolated using methods referenced in the Examples from sources including human cells and cells of *Drosophila*. Other sources include *E. coli*. The optimum concentration of recombinase to be utilized can be readily determined by one skilled in the art.

The invention also relates to a method of effecting DNA cleavage in a sequence-specific manner. At present, this technology is restricted by the

biological repertoire of available restriction endonucleases. In order to extend the range of sequence specific cleavages, several groups have attempted to direct cleavage to a target site by the use of homopyrimidine oligonucleotides bearing chemical cleavage groups for DNA. The rationale behind this approach is that triplex DNA formation is facile if the third strand consists exclusively of pyrimidines and the target duplex sequence consists of a "complimentary" stretch of homopurine and homopyrimidine strands. Once this three-stranded hybridization is effected, a chemical moiety such as Fe-EDTA or Cu-phenanthroline on the end of the third strand will carry out cleavage of the target duplex DNA. (See, Dreyer et al, 1985, *Proc. Natl. Acad. Sci USA* 82, 968-972).

The field of sequence specific cleavage is significantly extended by Applicants' discovery that a third strand of any sequence (i.e. one containing both purines and pyrimidines on one strand in any order) can form a triple helix with a homologous duplex sequence in the presence of recombination proteins. Using recombinase proteins to form a triple helix DNA containing a third strand oligonucleotide bearing a chemical cleavage group, (for example Fe-EDTA, Cu-phenanthroline or 2-methoxy-6-chloro-9-aminoacridine), cleavage of any duplex at any given sequence can be achieved. This technology can be expected to allow efficient sequence-specific cleavage of, for example, a 20 base sequence that occurs, for example, only once in the entire mammalian genome. One skilled in the art will appreciate that more frequent cutting of the genome can be achieved by manipulating the stringency of the recombinases for DNA homology (sequence identity

shared between the lignonucleotide and the duplex) to accommodate sequences that are similar but not identical, thereby directing cleavage to repetitive sequence motifs.

5 The ability to achieve sequence-specific cleavage at will has several implications. First, it provides an invaluable tool in the molecular cloning of DNA. Second, gene mapping over long stretches of DNA on the order of a million base pairs is rendered relatively
10 straightforward. Third, the ability to introduce a single cut in the mammalian genome, particularly when this is carried out within the cell, greatly facilitates gene targeting.

 The invention further relates to a method of
15 enhancing gene targeting. Gene targeting is an active field of study because it provides a method of constructing animal models of human disease and provides a mechanism by which gene therapy can be achieved (the correction of genetic disorders in
20 affected individuals). The ability to introduce sequence-specific breaks in genomic DNA using recombinase protein has the potential of overcoming the biggest obstacle in gene targeting to date, that is, the exceedingly low efficiency of targeting in cells.
25 One skilled in the art will appreciate that target sites for gene therapy include target sites for gene inactivation or RNA inactivation which abrogate gene expression.

 The invention further relates to a method of
30 identifying the presence of duplex DNA molecules of a specific sequence in a DNA-containing example. At present the identification of DNA molecules is oftentimes carried out by a laborious technique in which DNA molecules are electrophoresed on gels,

denatured, transferred to solid supports such as nitrocellulose membranes and probed or hybridized in situ to marker DNAs. According to this embodiment of the present invention, formation of a three-stranded DNA molecule can be effected in solution using recombinase proteins to carry out hybridization. The recombinase proteins can carry out hybridization of a labelled (e.g., radioactive) DNA probe to the duplex molecule of interest. The "tagged" molecule can then be visualized after separation from the excess non-hybridized probe by an appropriate technique (e.g., autoradiography following electrophoresis). Such an approach is faster, avoids denaturation of the molecule of interest, thus allowing study of the molecule in its native conformation, and permits the rapid screening of different molecules, for example, on a single gel, simply by using different probes.

The invention also relates to a method of using the formation of these three-stranded DNAs in cloning and mapping of DNAs having multiple restriction enzyme cleavage sites. It is oftentimes desirable to cleave at a limited subset of restriction sites. At present, hit and miss "partial cleavage" schemes are used or methylases are used that block cleavage at all sites recognized by certain restriction enzymes. Unfortunately, the repertoire of methylases is extremely limited and to the extent that they are available, result in the modification of all sites recognized by a particular restriction enzyme. Work from several labs has shown that triple helices involving polypurine/polypyrimidine sequences can abrogate cleavage by a restriction enzyme within the region of triple helix DNA. The technology disclosed herein, based on the use of recombinase proteins, can

extend such protection from cleavage to virtually any restriction enzyme recognition sequence.

Another aspect of the present invention relates to a method of using recombinase-formed three-stranded DNA to abolish transcription of a particular gene. At present, there is great interest in the use of "antisense" oligonucleotides to inhibit translation in cells. In this technique, oligonucleotides are hybridized in cells to messenger RNA (mRNA) to block synthesis (translation) of a particular protein, or to ribosomal RNA, thereby blocking all protein synthesis in the cell. This approach has severe limitations since all the available mRNAs corresponding to the protein of interest, or ribosomal RNA, must be targeted by the oligonucleotides, and there are often thousands of copies of the RNA that must be inactivated. The present invention provides a much more efficient approach in that transcription of only a particular gene is affected. In contrast to the large number of RNA transcripts for any single gene, the number of gene copies in cells is usually quite small, less than a dozen and usually not more than two copies per diploid cell. The present method of inhibiting protein synthesis by inhibiting gene expression is effected by targeting, by excess recombinase protein in a cell, of an oligonucleotide to a gene resulting in the formation of a DNA triple helix.

As noted above, the present invention relates to a method of effecting sequence-specific targeting of DNA. In general terms, the method utilizes the ability of a recombination protein, for example, the recA protein from *E. coli*, to pair an oligonucleotide to its homologous sequence in duplex DNA to form a three-stranded DNA molecule. A schematic of the formation of

such a synaptic complex and stable joint molecule is given in Figure 9.

The method to which the invention relates has wide applicability. It can be used to protect a specific sequence (that is, that sequence with which the oligonucleotide is complexed) from modification, for example, by a methylase, or, alternatively, from cleavage, for example, by a restriction enzyme. In addition, the present invention can also be used to effect site specific cleavage by attaching to the oligonucleotide a cleavage moiety, for example, a chemical cleavage moiety. Furthermore, the present method can also be used to accomplish site specific cleavage in a two-step process by, first, protecting the specific sequence from modification (by the formation of the three-stranded molecule) and, then, removing the oligonucleotide, thus making the prior-protected site available for cleavage (the other such sites being protected from cleavage by prior modification). In yet another embodiment, the present invention can be used to enrich a DNA pool for a desired sequence by derivatizing the oligonucleotide with one member of a binding pair, for example, biotin, and then selecting for the three-stranded molecule resulting from the complexation of the oligonucleotide with the desired duplex, using the other member of the binding pair, in this example, avidin. Purification of specific duplex DNA molecules can be accomplished in this manner. Other embodiments of the invention include the use of oligonucleotides linked to detectable labels for tagging specific duplex molecules, and the use of oligonucleotides bound to cross-linking reagents which, when activated, result in the formation of a stable three-stranded molecule.

Other applications of the present method will be clear to one skilled in the art from a reading of this disclosure.

Oligonucleotides suitable for use in the invention can be designed so as to optimize desired results. For example, the length of the oligonucleotides can be adjusted for particular targeting protocols without undue experimentation.

While the present invention will be described in some detail with reference to the above-described protection and restriction/modification embodiments, one skilled in the art will appreciate the broader applicability of this methodology both to in vitro and in vivo systems.

The protection aspect of the invention is described in some detail in Example 12 below. As will be clear from a reading of that Example, *recA* can be used to afford protection, for example, from cleavage, of a particular site in a duplex DNA molecule by effecting the formation of a synaptic complex at that site. Formation of the complex between the oligonucleotide and the homologous duplex DNA is both rapid and efficient.

For purposes of clarity, the specific sequence of reactions involved in the restriction/modification embodiment of the invention is detailed in Examples 13-15 below. For target DNA that is not easily sheared, such as lambda DNA which is 48.5 kilobases (kb) long (Daniels et al in *Lambda II*, Hendrix et al, Eds. (Cold Spring Harbor, N.Y. 1983), pp. 519-676), the reactions are advantageously done in solution. Larger genomes, however, can be embedded in agarose microbeads. (See Examples 14 and 15.)

The first step in achieving sequence-specific cleavage of duplex DNA, in the exemplified restriction/modification embodiment, is the selection of the particular Eco RI site for cleavage. A homologous oligonucleotide, generally 30 to 60 bases long, is synthesized such that the Eco RI site is, advantageously, centered in the oligonucleotide. Oligonucleotides having the recognition sequence at the 5' or 3' end can also be used, however, reduced efficiency may be observed (see Example 14). The oligonucleotide and recA protein are incubated with duplex DNA and the complex formed at the site of homology. Eco RI methylase and S-adenosylmethionine are then added and allowed to methylate all available sites, but spare the site involved in the oligonucleotide and recA protein complex. The complex and methylase are then inactivated, and Eco RI restriction enzyme is added to cleave at the now uncovered Eco RI site.

Cutting at a single site is depicted in Figure 17, however, two different oligonucleotides can be added at the same time. This allows isolation of a fragment from long or circular genomes. The following equation describes the yield of such a fragment:

$$\text{Yield (\%)} = (PC)^2 [1 - (1-M)C]^X (1-N) \times 100$$

where

- P is the efficiency of protection by recA protein of homologous sites from methylation (a value of 1 means complete protection),
- C is the efficiency of restriction enzyme cleavage,
- M is the efficiency of methylation of unprotected sites,

X is the number of Eco RI sites found in the fragment, and

N is the fraction of fragments destroyed by non-specific nucleases or shearing.

- 5 The three terms of the equation contain important parameters of the reaction. From the first terms, it is clear that protection of the homologous site should be maximized, and that drops in protection efficiency will be squared when two sites are involved.
- 10 Protection efficiencies are assumed to be the same for both sites. Because of the second term, which is raised to the X power, the methylation should advantageously, be carried very close to completion, especially for long fragments with multiple internal
- 15 Eco RI sites. From the third term, it is clear that nonspecific nucleases should be minimized, and, indeed, the use of proteins of high purity is preferred.

- One skilled in the art will appreciate from a reading of the foregoing that while the
- 20 oligonucleotides used can be underivatized, the versatility of targeting can be increased by derivatizing the oligonucleotide. Possible derivatives include proteins, biotin (as noted above), fluorescent dyes, chemically reactive moieties (for example, for
- 25 cleavage) or photochemically reactive moieties. Derivatization can be effected using methods known in the art.

- The method to which the invention relates has many applications, genomic mapping being one. The physical
- 30 distance between two loci is simply the fragment size. Once a fragment is isolated, for example, using pulsed field gel electrophoresis, the complexity of finding a particular desired gene is reduced several orders of

magnitude as compared to working with unfractionated genomic DNA.

One skilled in the art will appreciate from the foregoing that oligonucleotides can be designed to
5 target any site of a DNA sequence, including sites within large genomes. Accordingly, oligonucleotides can be designed which can be used in an intracellular milieu to target cleavage, recombination or repression of specific genes.

10 Certain aspects of the invention are described in greater detail in the non-limiting Examples that follow.

EXAMPLES

Experimental details relating to Examples 1-5.

Recombinas Proteins:

- Partially purified HeLa recombinase fraction was prepared from nuclear extracts as described elsewhere (Hsieh and Camerini-Otero, 1989, J.BioChem.264, 5089-5097). Partially purified Drosophila recombinase, fraction IV, was prepared from 24 hr embryos as described previously (Eisen and Camerini-Otero, 1988, *Proc Natl Acad Sci USA* 85, 7481-7485). The human recombinase preparation was free of any 3'-5' exonuclease activities as determined by trichloroacetic acid precipitable counts. No ³²P cpm were released from ³²P-3' end-labeled duplex DNAs after incubation with human recombinase fraction using joint molecule assay conditions (data not shown). As previously described, the Drosophila recombinase fraction removed <10% of ³²P cpm in a similar assay (Eisen and Camerini-Otero, 1988, *Proc Natl Acad Sci USA* 85, 7481-7485). Although not relevant to the joint molecule assays described here, 5'-3' exonuclease activity in recombinase fraction preparations were exceedingly low; <5% ³²P cpm and 10-15% ³²P cpm were released as trichloroacetic acid soluble counts by the Drosophila and human recombinase fractions, respectively (Eisen and Camerini-Otero, 1988, *Proc Natl Acad Sci USA* 85, 7481-7485). Purified *E. coli* recA and SSB proteins were generously provided by Dr. Stephen C. Kowalczykowski, Northwestern University Medical School (Kowalczykowski and Krupp, 1987, *J Mol Biol.* 193, 97-113). Both *E. coli* proteins migrate as a single homogeneous polypeptide by SDS PAGE and contain no detectable exonuclease activities as judged by release of trichloroacetic acid soluble counts (S.

Kowalczykowski, personal communication and data not shown).

DNA Substrates

- pGem 4 plasmid DNA was obtained from Promega
- 5 Biotec. M13mp18 viral DNA and restriction enzymes were obtained from New England Biolabs. M13mp19 viral DNA and pdel 9 plasmid DNA, a derivative of pBR322 deleted from nucleotides 1745 to 2505 (Brenner et al., 1985, MolCellBiol5, 684-691), were prepared according to
- 10 standard procedures. Oligonucleotides were synthesized and purified as described elsewhere (Hsieh and Camerini-Otero, 1989, JBioChem.264, 5089-5097). Oligonucleotides homologous to the plus strand of pdel 9 spanned pBR322 positions 17-29, 10-29 and 4359-29
- 15 (Sutcliffe, 1978, ColdSpringHarborSympQuantBiol43, 77-90) for the 13mer, 20mer and 33mer, respectively. Oligonucleotides homologous to the polylinker region of pGem 4 were derived from the negative strand of M13mp19 and mapped to M13mp19 positions listed below (Yannisch-
- 20 Perron, et al. 1985, Gene33, 103-119). Oligonucleotides used for partial duplexes spanned positions 6278-6290, 6265-6290, 6253-6290 and 6235-6290 for 13 bp, 26 bp, 38 bp and 56 bp respectively; those used for branch migration structures spanned 6278-6300,
- 25 6265-6300, 6253-6300 and 6235- 6300 for 13 bp, 26 bp, 38 bp and 56 bp, respectively. Oligonucleotides were labeled with ³²P-gamma-ATP (New England Nuclear) and T4 polynucleotide kinase (Pharmacia) and de-salted by passage over G25 spin columns (Boehringer Mannheim) or
- 30 by dialysis. Preparation of ³²P-labeled partial duplexes from labeled oligonucleotides and M13mp19 viral strand DNA was as described elsewhere (Hsieh and Camerini-Otero, 1989, JBioChem.264, 5089-5097). The branch migration structure was formed by incubating the

partial duplex molecule with an unlabeled

ligonucleotide identical in sequence to the ³²P-labeled
annealed oligonucleotide, but containing an additional
10-base annealing site immediately 5' to the ³²P-labeled
5 fragment. DNA concentrations are expressed as moles of
nucleotides or by weight.

The length of homology shared between a single-
strand and double-strand substrate is defined as the
maximum number of bases that could be paired between
10 the single-strand DNA and the complementary strand of
the linear duplex substrate in a joint molecule. Two
significant regions of homology shared between pGem 4
and M13mp18 were found using the Univ. of Wisconsin
BESTFIT program. They are in opposite orientations
15 with respect to each other in pGem 4. Map positions
for the 59 bp region from the lac i gene are: M13mp18
6001-6059 (Yannisch-Perron et al., 1985, Gene 33, 103-
119) and pGem 4 104-162. The 57 bp polylinker region
maps to M13mp18 positions 6231-6287 and in pGem 4 to
20 positions 10-66.

pGem 4 Linear Duplex with a Unique 3' ³²P-label

pGem 4 DNA was linearized with Hind III and labeled
by filling in 3' ends with ³²P-alpha-dATP (New England
Nuclear) and cold deoxynucleotides using the Klenow
25 fragment of DNA polymerase (Pharmacia). The reaction
was stopped by heating at 65°C for 10 min, was brought
to 100 mM NaCl and digested with Bgl I. The 1,635 bp
Bgl I-Hind III fragment containing the polylinker
region was gel purified and dialyzed extensively
30 against 10mM Tris-HCl, pH 8.0 and 1mM EDTA. When this
³²P-labeled fragment was digested with Pst I and
analyzed by densitometry after electrophoresis on an 8%
denaturing polyacrylamide gel or a 1% agarose gel, >99%
of the label was removed from the 1.6 kb fragment. The

³²P- label thus resided within 10 bases of the 3' end of the plus strand of the duplex substrat .

Joint Molecule Assays

Joint molecule assays, 20 ul, were carried out for
5 10 min at 37°C as described (Hsieh and Camerini-Otero,
1989, J.BioChem.264, 5089-5097) using 150 pmol (50 ng)
of M13mp18 viral DNA and 150 pmol (50 ng) linear pGem 4
DNA or 300 pmol (100 ng) linear pdel 9 and 5 ng ³²P-
labeled oligonucleotide with 100 ng HeLa protein or 40
10 ng Drosophila protein. Assays containing 7 ug reca
protein and 700 ng SSB protein were carried out for 10
min at 37°C with preincubation as described (Hsieh and
Camerini-Otero, 1989, J.BioChem.264, 5089-5097).
Assays were brought to 1% SDS and 10 mM EDTA prior to
15 electrophoresis at room temperature on 0.7% agarose
gels in TAE buffer (40 mM Tris-acetate, pH 8, 1 mM
EDTA) containing ethidium bromide for 12-16 hr at
0.6V/cm. Quantitation was accomplished by
densitometry. Electron microscopy was performed using
20 the modified Kleinschmidt technique.

Thermal Stability Assays

³²P-labeled partial duplexes (150 pmol) consisting
of a ³²P-labeled oligonucleotide annealed to M13mp19
viral strand DNA were incubated for 10 min at the
25 indicated temperatures or on ice in strand exchange
buffer containing 1% SDS and 10 mM EDTA. TAE buffer
containing 0.1% bromophenol blue and 50% glycerol was
added and the mixtures were electrophoresed for 12- 16
hr at 0.6V/cm on 0.7% agarose gels in TAE buffer
30 containing ethidium bromide followed by
autoradiography. For stability assays of branch
migration structures, 150 pmol of the ³²P-labeled
partial duplexes and 4 ng of a second oligonucleotide
containing a 10 base annealing site to M13mp19 were

incubated for 10 min at the indicated temperature or on ice in strand exchange buffer containing 1% SDS, 10 mM EDTA and 10% w/v poly thyl ne glyc l. The reactions were electrophoresed on agarose gels followed by

5 autoradiography as described above. Joint molecules were formed by recombinases. The reactions were stopped in 1% SDS and 10mM EDTA, incubated an additional 10 min at the indicated temperatures or on ice and electrophoresed on agarose gels.

10 Deproteinizing Joint Molecules

Reacted joint molecule assays containing recA and SSB proteins were brought to 1% SDS, 10mM EDTA and 20 ug/ml Proteinase K (Boehringer Mannheim) and incubated for an additional 20 min at 37°C. The assays were

15 extracted sequentially with phenol, phenol-chloroform and chloroform. The samples were extracted four times with anhydrous ethyl ether saturated with water just before use. Residual ether was removed under nitrogen. The deproteinized joint molecules were analyzed by SDS

20 PAGE (Laemmli, 1970) on 12% polyacrylamide gels (Novex) followed by silver staining (Hochstrasser et al., 1988, AnalBiochem 173, 412-423) or Western blotting. Electrophoresis and transfer to nitrocellulose using a Schleicher and Schuell Miniblot apparatus were

25 according to manufacturer's directions. Following transfer, the nitrocellulose filters were blocked in 10% milk in phosphate buffered saline, PBS, for 1 hr. The filters were incubated for 1 hr with 20 ml of a 1:500 dilution of rabbit antisera raised to purified

30 recA protein (Hazleton Biotechnology) in 5% milk, 0.1% v/v Tween 20, in PBS. After extensive washing, the filters were incubated for 1 hr with 20 ml of 5% milk, 0.1% Tween 20 in PBS containing 15 µCi of ¹²⁵I- labeled donkey anti-rabbit IgG (3000 Ci/mmol, Amersham)

followed by extensive washing. Autoradiography was for 3 days using intensifying screens.

Example 1. Joint Molecules with Short Regions of Homology

- 5 To test for the formation of three-stranded DNA, recombinase proteins were monitored for their ability to form stable joint molecules with short regions of homology. Recombinase proteins were incubated with a linear duplex DNA and a homologous circular single-
- 10 strand DNA to form a joint molecule product in which the two substrates are noncovalently joined. Stable joint molecule formation initiates from the ends of the linear duplex. The circular single-strand DNA substrate is the viral (plus) strand of M13mp18 phage.
- 15 The linear duplex substrate is a plasmid, pGem 4 (Fig. 2A). pGem 4 and M13mp18 share two significant regions of homology, a 59 bp region from the *E. coli lac i* gene and a homologous 57 bp region that constitutes the polylinker cloning sites of both pGem 4 and M13mp18.
- 20 These two homologous regions are separated in pGem 4 by 37 bp of nonhomologous sequence. When pGem 4 is linearized in the polylinker region, the polarity of strand exchange by the human (HeLa) and *Drosophila* recombinase fractions dictates that only homologous
- 25 sequences at the right end of the pGem 4 linear duplex as shown in Fig. 2A are utilized (Hsieh et al., 1986, Cell 44, 885-894; Eisen and Camerini-Otero, 1988, Proc Natl. Acad Sci USA 85, 7481-7485). The right end of pGem 4 as drawn exposes the 3' end of the plus strand of the
- 30 M13mp18 polylinker sequence.

HeLa recombinase fraction formed joint molecules between M13mp18 single-strand DNA and linearized pGem 4 double-strand DNA (Fig. 2B). The samples were deproteinized prior to electrophoresis. Surprisingly,

as few as 13 bases of shared homology was sufficient for the formation of stable joint molecules (lane 4). Homologous sequences five bases long on the right end of the linear duplex and 52 bases long on the left end of the duplex were not utilized (lane 5). This result confirms the directionality of strand exchange by the human recombinase fraction. Control experiments using M13mp19 single-strand DNA containing the negative strand of the polylinker region resulted in the appearance of stable joint molecules when pGem 4 was linearized with Eco RI but not Hind III. Deletion of all but 5 bp of homology in assays using pGem 4 digested with both Hind III and Eco RI (lane 6) also yielded no product indicating that the second region of shared homology located at an internal site 37 bp from the left end of the duplex is not utilized by the HeLa recombinase fraction in forming joint molecules. The joint molecule assay using very short sequence homologies was surprisingly efficient with 16-26% of the duplex converted to joint molecules. Under similar assay conditions using completely homologous DNAs, human recombinase fraction converts one-third to one-half of the linear duplex to joint molecules (Hsieh, et al., 1986, Cell 44, 885-894; Hsieh and Camerini-Otero, 1989, J.Biochem. 264, 5089-5097).

Like the human recombinase fraction, the *Drosophila* recombinase fraction also formed joint molecules with as few as 13 bp of homology (Fig. 2C lane 4), did not utilize 5 bp of homology and exhibited the expected directionality of joint molecule formation (lane 5). Approximately 20% of the linear duplex was converted to joint molecules in lanes 1-3. Under similar assay conditions using completely homologous DNAs, *Drosophila* recombinase fraction converts two-thirds of the duplex

to joint molecules (Eisen and Camerini-Otero, 1988, Proc. Natl Acad Sci USA 85, 7481-7485).

Joint molecule assays using pGem 4 linear duplex, M13mp18 single-strand DNA, recA protein and E. coli SSB protein are shown in Fig. 2D. Assay conditions were those that have been shown by others to promote extensive strand exchange by recA over thousands of basepairs (reviewed in Radding, 1982 Ann Rev Genet 16 405-437). It was observed that 56 or 38 bp of homology (lanes 1 and 2) was sufficient for the formation of joint molecules by recA; 28% and 19% of the linear duplexes were converted to joint molecules, respectively. However, no joint molecules were observed when the homology was limited to 26 bp (lane 3). Under identical reaction conditions using completely homologous substrates, recA was observed to convert essentially all of the duplex to higher order structures. In this assay, the directionality of recA was identical to that of the eukaryotic recombinase fractions, i.e., only homology on the right end of the linear duplex was utilized by recA protein (compare lanes 1 and 5). As observed for the human recombinase fraction, joint molecules were formed efficiently with Eco RI-linearized pGem 4 but not with Hind III-linearized duplex when recA and SSB proteins were incubated with M13mp19 single-strand DNA. At present, it is unknown why the polarity of strand pairing by recA with short regions of homology appears different from that observed by others with substrates sharing extensive regions of homology (reviewed in Radding, 1982). It has been observed, however, that the directionality of strand exchange by recA, unlike that of the eukaryotic recombinases, is substrate-dependent (Konforti and Davis, 1990, J Bio Chem. 265, 6916-6920).

Electron microscopy of joint molecules formed by *recA* confirmed that the pairing of a linear duplex to one single-strand circular DNA occurred at the end of the duplex. Although no displaced strand was seen, the resolution was insufficient for determining the configuration of the third strand in the region of pairing. In addition, the formation of joint molecules by all three recombinases required the simultaneous presence of both DNAs and recombinase protein and was not due to exonucleolytic processing of the duplex and subsequent annealing of the single-strand DNA (see Fig. 6). When M13mp18 single-strand DNA and Hind III-linearized pGem 4 DNA (56 bp of homology) were separately incubated with recombinase followed by co-incubation of the treated substrates at 65°C for 10 min in the presence of 1% SDS and 10% w/v polyethylene glycol to promote nonenzymatic annealing no joint molecules were formed.

Example 2. A Novel Joint Molecule Assay

An explanation for the stability of these joint molecules is that the circular single strand is nonspecifically held in place or "pinched" by the duplex at the junction of the region of homology and nonhomology. To rule this and other forms of trapping out, a novel assay was designed in which the single strand has two ends, is very short and is completely homologous to the duplex. Furthermore, this assay demonstrates that recognition of short homologies is not limited to the polylinker sequence of M13mp18. A novel assay for the formation of joint molecules between a linear duplex DNA and a 5'-³²P-labeled oligonucleotide identical to the 3' end of one strand of the linear duplex is shown in Fig. 3A. The formation of stable joint molecules was monitored by

the appearance of label migrating at the position of linear duplex DNA on agarose gels.

Stable joint molecules were formed by the human recombinase fraction between p ϕ 19 linear duplex DNA and an oligonucleotide 33 bases or 20 bases in length (Fig. 3B, lanes 1 and 3). No joint molecules were formed with an oligonucleotide 13 bases long (lane 5) or with a nonhomologous oligonucleotide. Approximately 50% of the linear duplex in lane 1 was converted to joint molecules. Control experiments in lanes 2, 4 and 6, demonstrate that joint molecules were not formed by degradation of the duplex by potential exonucleases in the recombinase fraction preparations thereby exposing the duplex strand that could anneal to the oligonucleotide; when the two DNA substrates were separately incubated with recombinase fraction and then co-incubated for 10 min at 65°C in the presence of SDS to stop activity, virtually no joint molecules were observed. No joint molecules were formed when an oligonucleotide corresponding to the negative strand of p ϕ 19 was used, reflecting the 3'-5' directionality of the human recombinase fraction, i.e. pairing of the oligonucleotide initiates at the 3' end of the noncomplementary strand of the duplex.

25 Example 3. Thermal Stability of Joint Molecules

If the deproteinized joint molecules with short regions of homology discussed above had a displaced strand, branch migration would lead to their rapid dissociation. DNA branch migration is a random walk process where, depending on the geometries of the DNAs examined, the time required to step through a base pair is between 12-200 microseconds (Thompson, et al., 1976, *Proc Natl Acad Sci USA* 73, 2299-2303; Radding, et al. 1977, *J. Mol Biol* 116, 825-839; Green and Tibbetts, 1981, *Nucl Acids*

Res 9, 1905-1918). Thus, the time required to reach the end of the duplex is approximately equal to the distance (56 bp) squared multiplied by the step time (0.2 msec) divided by 2 or about 300 milliseconds (Feller, 1957, Vol. 1. p. 325, John Wiley & Sons, New York). Surprisingly, these deproteinized joint molecules were observed to be stable for hours ($>10^4$ sec) at room temperature.

The stability of joint molecules at room temperature reflects the formation of additional interactions between the third strand and the duplex. In order to ascertain the strength of these interactions and to rule out covalent interactions, the thermal stability of joint molecules was examined and compared with the stabilities of partially duplex molecules and molecules that can undergo non-enzymatic branch migration (see Fig. 4A). The short duplex regions of the partially duplex molecule, I, corresponded exactly in sequence and length to the presumed paired regions of joint molecules formed by recombinase between pGem 4 linear duplexes digested with Hind III (56 bp), Sal I (38 bp), Bam HI (26 bp) and Kpn I (13 bp) and M13mp18 single-strand DNA shown in Figure 2. The branch migration structure, II, corresponded exactly in sequence, length and direction of potential branch migration to the joint molecules formed by recombinases (see Materials). Non-enzymatic branch migration would result in the displacement of the ^{32}P -labeled oligonucleotide. In this assay, displacement of the annealed fragment was via homology-dependent branch migration since no displacement of a ^{32}P -labeled fragment was observed when the second oligonucleotide contained a 10 base annealing site

attached to a sequence not homologous to the duplex region.

Repr sentative data from stability experiments for a homologous region of 38 bp corresponding to joint molecules formed between Sal I-linearized pGem 4 and M13mp18 DNAs are shown in Fig. 4B. While the duplexes (58% G-C) were stable after 10 min at 65° C, melting at 75°C, branch migration structures dissociated after 10 min at 45°C. Joint molecules formed by human recombina-
10 se fraction were stable after 10 min at 65°C and dissociated at 75°C. This represents a 30°C difference between the relative stability of joint molecule products formed by recombinase versus branch migration structures of identical length, sequence and
15 direction of branch migration. The faint band migrating slower than joint molecules in the ethidium bromide-stained gel is a dimer of pGem 4 resulting from a contaminating ligase activity.

Similar thermal stability data for duplexes, branch migration structures and joint molecules formed by the
20 three recombinases over four different lengths of homology are summarized in Fig. 5. Joint molecules were exceedingly stable dissociating at temperatures 20 to 30 degrees higher than those of branch migration
25 structures. In all cases, observed melting temperatures of the duplexes were in close agreement with calculated T_m 's based on sequence length and G-C content (Sambrook, et al., 1989 Molecular Cloning Laboratory
Manual Cold Spring Harbor Press Cold Spring Harbor). In addition, the
30 observation that dissociation temperatures for joint molecules are proportional to the length of homology makes it highly unlikely that a covalent bond is responsible for the stability.

Joint molecules were not stabilized by nonspecific trapping or pinching of the DNA substrates resulting from the use of a large, circular single-strand DNA, e.g. a joint molecule having a structural block right at the exchange point flanked by normal duplex DNA (see also Fig. 3). Joint molecules formed by human recombinase fraction between an M13mp18 linear duplex and a homologous ³²P-labeled oligonucleotide 26 bases long were also significantly more stable than corresponding branch migration structures. Deproteinized joint molecules dissociated at 55° C as did the corresponding duplexes (42% G-C) while branch migration structures were unstable at room temperature (data not shown).

Reconstruction of joint molecule structures nonenzymatically was performed in order to assess their stability relative to joint molecules formed by recombinases. Heat-denatured linear pGem 4 and M13mp18 single-strand DNA were annealed at neutral pH or at pH 5.5 (Voloshin, et al., 1988, Nature 333, 475-476) in the presence of 10% w/v polyethylene glycol and electrophoresed on agarose gels. No joint molecules were observed. Similarly, annealing heat-denatured linear pdel 9 with ³²P-labeled oligonucleotides failed to yield stable joint molecules.

Example 4. Joint Molecules That Are Stable Have Three Intact Strands

The surprising stability of these joint molecules could be explained if the 3' end of the plus strand of the linear duplex had been exonucleolytically degraded leaving only heteroduplex DNA in the joint molecule (see Fig. 1, top). The following experiment shows that stable joint molecules have an intact third strand (the plus strand of the polylinker region).

Joint molecule assays using HeLa recombinase fraction or recA and SSB proteins were carried out between M13mp18 single-strand DNA and a fragment of pGem 4 ³²P-labeled at the extreme 3' end of the plus strand of the linear duplex (Fig. 6A). Restriction enzyme digests of the labeled duplexes confirmed that >99% of the ³²P-label was restricted to the 3' terminal 10 bases of the plus strand of the pGem 4 fragment (see Methods). Thus, formation of ³²P-labeled joint molecules would indicate that the third strand is intact since heteroduplexes of 9 bp or less are very unstable and impossible to recover under these conditions (cf. Fig. 5). Since 15-20% of the labeled duplex was converted to joint molecules in these assays, virtually all of the ³²P-labeled joint molecules must have intact third strands. The thermal stability of ³²P-labeled joint molecules formed by recA (Fig. 6B) indicated that molecules having three intact strands dissociated at relatively high temperatures (cf. Fig. 5). The rapidly migrating lower band represents the nonenzymatic loss of ³²P-label from the ³²P-labeled duplex which occurred at higher temperatures in the absence of recombinase protein.

Quantitation of the relative stabilities of ³²P-labeled joint molecules formed by recA and HeLa recombinase fraction and the corresponding 56 base branch migration structure is shown in Fig. 6C. The ³²P-labeled joint molecules formed by human recombinase fraction, like those formed by recA protein, were exceedingly stable. These results using a truncated pGem 4 linear duplex also demonstrate that the stability of joint molecules derives from pairing restricted solely to the region of homology in the polylinker at the right end of the linear duplex.

Example 5. Stable Joint Molecules Are Devoid of Protein

A possible explanation for the stability of joint molecules is that they remain associated with recombinase protein in the presence of 1% SDS. The example depicted in Fig. 7, demonstrates that the stable DNA joint molecules formed by recA protein are devoid of any associated recA molecules after treatment with several deproteinizing agents. Joint molecules having 56 bp of homology were deproteinized with Proteinase K in the presence of 1% SDS and extracted with phenol/chloroform as described above. The level of residual recA protein was assessed by Western blotting using a polyclonal antibody against recA protein (Fig. 7A). Identically treated joint molecules were also analyzed for thermal stability and dissociated between 75° and 85°C (Fig. 7B).

Based on the experimental limits of detection of recA protein by Western blotting (0.1 ng or 2.5 fmol, Fig. 7A lane 5), and the recovery of deproteinized joint molecules (30 fmol, Fig. 7B lane 3), it is estimated that greater than 90% of the joint molecules were free of recA protein (Fig. 7A lane 6). No detectable recA polypeptides were observed by silver staining. In the blot, no recA was observed migrating at the position of recA polypeptides or single-strand or double-strand DNA.

It was also observed that even if active recombinases were present, they did not interfere with branch migration and subsequent dissociation of the DNA molecule. The ³²P-labeled partial duplex structures shown in Fig. 4A were incubated at 37°C with a second oligonucleotide containing a 10 base annealing site in the presence of recA and SSB proteins or HeLa

rec mbinase fracti n under joint molecu l assay conditions. Branch migrations structures were formed followed by displacement of the ^{32}P -labeled oligonucleotide within 10 minutes.

5

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Experimental details relating to Examples 6-12.

RecA protein:

Purified E. coli recA protein was provided by Dr. Stephen C. Kowalczykowski, Northwestern University

10 Medical School.

DNA substrates:

pBR322 and pUC18 plasmid DNAs and M13mp18 replicative form DNA were from Pharmacia.

Oligonucleotides were synthesized and purified by
15 passage over a Mono Q column (Pharmacia) as described previously (Hsieh and Camerini-Otero, J. Biol. Chem. 264:5089 (1989)). Oligonucleotides were 5'-end labeled with ^{32}P -gamma-ATP (New England Nuclear) and T4 polynucleotide kinase (Pharmacia) as described
20 previously (Hsieh and Camerini-Otero, J. Biol. Chem. 264:5089 (1989)).

Oligonucleotides completely homologous to the plus strand of pBR322 (Figures 11 and 14) spanned pBR322 positions 15-29, 10-29, and 4359-29 for the 15, 20 and
25 33 base oligonucleotides, respectively (Sutcliffe, *Cold Spring Harbor Symp. Quant. Biol.* 49, 561 (1978)). The 20L series of oligonucleotides (Figure 15) had varying amounts of homology to the plus strand of pBR322 at the 3' end and spanned positions 10-29, 20-29, 22-29, 24-
30 29, and 26-29 for 20, 10, 8, 6, and 4 bases, respectively. The 20R series (Figure 15) had homology to the plus strand of pBR322 at the 5' end and spanned positions 20-39, 20-29, 20-27, 20-25, and 20-23 for 20, 10, 8, 6 and 4 bases, respectively. Oligonucleotides

homologous to the plus strand of pUC18 (Figure 10) spanned positions 230-285, 230-267, 230-255, and 230-249 for the 56, 38, 26 and 20 base oligonucleotides, respectively (Norrander et al, *Gene* 26, 101 (1983)).

- 5 The 33-base oligonucleotide homologous to positions 4359-29 of pBR322 was not paired to the polylinker region of pUC18. The 20 base oligonucleotide used in the experiment shown in Figure 12 was homologous to positions 248-267 of the negative strand of pUC18. The
10 oligonucleotides shown in Figure 13 contained 56 bases homologous to the plus strand of pUC18 corresponding to positions 230-285. The 30 base oligonucleotide homologous to the plus strand of M13mp18 (Figure 16) spanned positions 6831-6860 (Yannisch-Perron et al, *Gene*
15 33, 103 (1985)). DNA concentrations are expressed as moles of nucleotide or by weight.

Synaptic complex formation:

- Synaptic complexes were formed by incubating 1.8 μ M (15 ng) oligonucleotide, 18 μ M duplex DNA (150 ng) and
20 1.5 μ M (1.5 μ g) recA protein in a buffer containing 20 mM Tris-HCl, pH 7.5, 0.4 mM dithiothreitol, 12.5 mM MgCl₂, 0.3 mM ATP-gamma-S (Fluka) and 1.1 mM ADP (Sigma) in a total volume of 25 μ l for 15 min at 37°C.

- Following synaptic complex formation, 10-20 units of
25 the appropriate restriction endonuclease (New England Biolabs) were added and incubation continued for an additional 5 min. The reaction was quenched by the addition of SDS and EDTA to a final concentration of 1% and 10 mM, respectively. Reactions were

- 30 electrophoresed on 1% agarose gels in 40 mM Tris-acetate, pH 8.0, 1 mM EDTA and 1 μ g/ml ethidium bromide at 0.6 V/cm for 14-16 h at room temperature.

Quantitation was determined by comparison of reacted assays with 150 ng of unreacted duplex DNA using

densitometer scanning of Polaroid 665 negatives.

Recoveries of intact duplex DNA in synaptic complex assays were compared to a standard containing 150 ng unreacted duplex DNA. In some cases, synaptic complex assays were quenched by the addition of 1% SDS and electrophoresed on 1% agarose gels in 89 mM Tris-borate, pH 8.3, 5 mM MgCl₂ at 0.6 V/cm for 14-16 h at room temperature.

Deproteinized joint molecules:

- 10 Synaptic complexes were formed as described above except that 5'-³²P-labeled oligonucleotides were used. The reactions were quenched by the addition of SDS and EDTA and electrophoresed as described. The gels were then fixed and exposed on Kodak XAR-2 film. In some cases, joint molecules were deproteinized by proteinase K (Boehringer Mannheim) treatment and phenol:chloroform extraction as described previously prior to electrophoresis (Hsieh et al, *Genes Devel.* 4, 1991 (1990)). Quantitation of joint molecules was determined by a reconstruction experiment in which a ³²P-labeled 56-base oligonucleotide was annealed to known quantities of M13mp19 single-strand DNA at 65°C or in the presence of recA. (No difference was observed in the efficiency of annealing.) Following electrophoresis and autoradiography, the relative intensities of these annealed standards were compared with those of joint molecules.

EXAMPLE 6

Formation of Synaptic Complexes and Joint Molecules

- 30 The experimental scheme for the formation of synaptic complexes and stable joint molecules by recA is shown in Figure 9. Formation of synaptic complexes is accomplished by incubating a duplex DNA such as a supercoiled plasmid DNA, a homologous oligonucleotide

and recA protein. The oligonucleotide spans a restriction endonuclease recognition site in the duplex DNA. Formation of a synaptic complex involving recA protein, oligonucleotide and the duplex DNA renders the duplex resistant to cleavage by the restriction endonuclease. The restriction endonuclease footprint corresponding to a synaptic complex can be visualized on ethidium bromide-stained agarose gels as supercoiled plasmid DNA remaining after incubation of complexes with the appropriate restriction endonuclease. RecA protein can be dissociated from these synaptic complexes by adding EDTA and SDS detergent, and deproteinized joint molecules result in which the oligonucleotide (5' end-labeled with ^{32}P) is stably paired with the duplex. The presence of joint molecules can be determined by assaying for the appearance of ^{32}P label migrating on agarose gels at the position of duplex DNA.

The formation of joint molecules by recA in the presence of ATP and an ATP regenerating system was examined. It had previously been observed that, under these reaction conditions, stable, deproteinized joint molecules were formed by recA between a linear duplex and a single-strand circular DNA sharing less than 60 bp of homology (Hsieh et al, *Genes Devel.* 4, 1991 (1990)). Stable, deproteinized joint molecules were formed by recA between pUC18 supercoiled DNA and a homologous 56-base oligonucleotide. In the presence of ATP hydrolysis, deproteinized joint molecules were recovered after 1 min but were very unstable; after 3 min, half of the joint molecules had dissociated, and after a 15 min incubation, no deproteinized joint molecules were recovered. Once the initial round of pairing and dissociation had occurred, it appeared that

the duplex was unable to participate in additional rounds of pairing. Due to the transient nature of this pairing, footprinting of synaptic complexes was not possible in the presence of ATP. It was observed that replacement of ATP with a nonhydrolyzable analogue of ATP, ATP-gamma-S, and ADP allowed freezing of the pairing reaction and accumulation of intermediates.

The formation of synaptic complexes and stable joint molecules by recA protein in the presence of 0.3 mM ATP-gamma-S and 1.1 mM ADP is shown in Figure 10. ³²P-labeled oligonucleotides homologous to pUC18 plasmid polylinker sequences were incubated with supercoiled pUC18 plasmid DNA in the presence of recA protein followed by the addition of Sac I restriction endonuclease. As depicted in Figure 10A, all the oligonucleotides spanned a unique Sac I restriction endonuclease recognition site in the pUC18 plasmid.

Synaptic complexes were formed with 20 bases of homology shared between the oligonucleotide and the duplex DNA (see Figure 10B, lane 9). Quantitation of the amount of supercoiled DNA protected from digestion by Sac I indicated that 70-75% of the duplex DNA was present as synaptic complexes when the oligonucleotide contained 56 or 38 bases of homology (lanes 3 and 5). Fifty-five percent and 20% of the duplex were converted to synaptic complexes with 26 and 20 bases, respectively, of homology. Formation of synaptic complexes required the presence of both recA protein and the homologous oligonucleotide. A control in lane 1 indicates that when the duplex was incubated with oligonucleotide and recA, but without restriction enzyme, the supercoiled DNA remained intact. As shown in lane 10, the footprint of the synaptic complex did not extend appreciably beyond the region of the duplex

that is colinear with the oligonucleotide since synaptic complexes do not afford protection from cleavage by Hind III which cleaves at a site located 14 bp from the region spanned by the 38-base

- 5 oligonucleotide. In addition, in this assay, a nonhomologous oligonucleotide does not result in the formation of a synaptic complex by recA.

- RecA can form joint molecules that are stable when deproteinized between a homologous ³²P-labeled
10 oligonucleotide and pUC18 plasmid DNA (Figure 10C). As few as 26 bases of homology shared between the oligonucleotide and the duplex DNA is sufficient for the formation of joint molecules in this assay whereas twenty bases of homology is not sufficient.
15 Quantitation of the recovery of stable joint molecules indicates that approximately 20-50% of the pUC18 duplex was paired with a homologous oligonucleotide 56 bases long when the complexes were electrophoresed in TAE buffer (see description of Figure 10). As was observed
20 for synaptic complexes, the efficiency of joint molecule formation by recA increases as a function of the length of homology available for pairing. These deproteinized joint molecules dissociate when the superhelical strain is relieved upon linearization at a
25 restriction endonuclease site located outside the region of pairing (Figure 10C, lane 10).

- The joint molecules formed between a duplex DNA and an oligonucleotide are not stabilized by residual recA protein. When synaptic complexes were deproteinized by
30 treatment with SDS, proteinase K and phenol/chloroform extraction, the number of stable joint molecules recovered was unchanged.

The assay conditions used for the formation of synaptic complexes were those that proved optimal for

joint molecule formation with 56 bp of homology. The formation of joint molecules exhibited a sharp optimum for an oligonucleotide concentration of 1.2 μM , with 0.9 μM oligonucleotide yielding no joint molecules; increasing the oligonucleotide concentration to 1.8 μM resulted in no further increase in the yield of joint molecules. The amount of recA used in these assays (1.5 μM) is saturating with respect to the single-strand oligonucleotide concentration. The ready detection of synaptic complexes and joint molecules was dependent on the presence of both ATP-gamma-S and ADP in a 1:3 molar ratio. Alteration of the ratio of ATP-gamma-S to ADP or the concentration of these two cofactors reduced the efficiency of both synaptic complex and joint molecule formation.

It is well established that divalent cations are essential for recA activity in vitro. A study was undertaken to determine whether Mg^{2+} is required to stabilize joint molecules. Synaptic complexes were formed in the presence of recA as described in Figure 10. The reactions were deproteinized by the addition of SDS alone and the reaction products analyzed by electrophoresis on agarose gels containing 5mM MgCl_2 . Although the recovery of joint molecules in the presence of Mg^{2+} was 2-4 fold higher than when Mg^{2+} was omitted from the electrophoresis step, no qualitative differences were observed, i.e., stable joint molecules were formed with oligonucleotides containing 26 bases but not 20 bases of homology.

The data presented in Figure 10 indicate that the formation of synaptic complexes in the presence of ATP-gamma-S is an intermediate step in the pathway leading to the formation of stable, deproteinized joint molecules. The formation of deproteinized joint

molecules and synaptic complexes containing recA exhibit a dependence on the length of homology available for pairing. Also, the formation of both synaptic complexes and joint molecules occurs with relatively high efficiency for 56 bases of homology; that is, upon deproteinization of these synaptic complexes, most of the duplex DNA is still paired with the oligonucleotide in stable joint molecules in the presence of Mg^{2+} .

10

EXAMPLE 7

Synaptic Complexes Containing Linear Duplex DNA

Superhelical strain is not essential for the formation of synaptic complexes involving very short regions of homology. The formation of synaptic complexes involves recA, a linear duplex and a homologous oligonucleotide 33 bases long (Figure 11A). In Figure 11B, it is readily seen that recA formed synaptic complexes between these two substrates resulting in protection of the linear duplex from cleavage by Cla I (lane 4). In the absence of either recA or oligonucleotide (lanes 2 and 6, respectively) or in the presence of a nonhomologous oligonucleotide (lane 5), synaptic complexes were not formed.

25

EXAMPLE 8

Footprinting Synaptic Complexes

The extent of protection from restriction endonuclease cleavage conferred by a recA synaptic complex was mapped (Figure 12). A 20-base oligonucleotide homologous to a region in the polylinker sequence of pUC18 plasmid DNA was incubated in the presence of supercoiled pUC18 and recA to allow the formation of synaptic complexes. Protection was seen at sites for Bam HI, Kpn I, Pst I, Sac I and Sph I restriction endonucleases. However, cleavage by Eco RI

30

and Hind III was unimpaired by the presence of the synaptic complex. Accordingly, the footprint of the synaptic complex apparently extends approximately 13-14 bases beyond the 5' and 3' ends of the paired oligonucleotide and is symmetrical.

EXAMPLE 9

Directionality of Synaptic Complex and Joint Molecule Formation

The apparent directionality of joint molecule formation is influenced by the choice of DNA substrates, the length of shared homology and the relative stabilities of joint molecules formed with opposite polarities (Konforti et al, *J. Biol. Chem.* 265, 6916 (1990); Rao et al, *Proc. Natl. Acad. Sci.* 88, 2984 (1991)). Therefore, the directionality of both synaptic complex formation and joint molecule formation involving a supercoiled duplex and an oligonucleotide was examined. The formation of synaptic complexes involving 56 bp of homology does not exhibit directionality. Synaptic complexes were formed by recA regardless of the positioning of the homologous sequence with respect to the ends of the oligonucleotide. In the experiment in Figure 13, a 56-base oligonucleotide homologous to the polylinker region of pUC18 was used or one of several other oligonucleotides having the same 56-base sequence plus 20 bases of nonhomologous sequence at the 5' end, at the 3' end or at both the 5' and 3' ends of the oligonucleotide (Figure 13A). In all cases, synaptic complexes were formed with about equal efficiencies (Figure 13B, lanes 5, 7, 9, 11). In contrast, formation of stable joint molecules by recA exhibited polarity showing a strong preference for homology at the 5' end of the oligonucleotide; the number of joint

molecules formed with the 76R oligonucleotide was half as many as with the 56-base oligonucleotide (Figure 13C, lanes 2 and 6) whereas the 76L oligonucleotide (lane 4) or 96 oligonucleotide (lane 8) yielded tenfold fewer joint molecules.

EXAMPLE 10

Minimum Structure Required for the Homology Search

Three oligonucleotides, 33, 15 or 13 bases long and homologous to a pBR322 sequence (Figure 14A) were incubated with pBR322 supercoiled plasmid DNA in the presence of recA. Potential cleavage sites for the 15 base oligomer are shown in Figure 14B and the footprint is shown in Figure 14C. The 15 base oligomer was of sufficient length to form synaptic complexes as evidenced by resistance to cleavage by Hind III and Cla I endonucleases (lanes 4 and 7). In this assay, use of a 33-base oligomer resulted in the formation of synaptic complexes, but a 13-base oligomer did not. Control experiments indicate that the formation of synaptic complexes required the presence of both oligonucleotide and recA. The footprint of the synaptic complex did not extend appreciably beyond the region of pairing (see Figure 14C, lane 10). This experiment also demonstrates that formation of synaptic complexes by recA was not restricted to any particular sequence since recA paired homologous DNAs containing either a pUC18 (Figure 10) or a pBR322 sequence (Figure 11).

50.

EXAMPLE 11

Nucleation of Pairing Involves One-Half
of a Helical Turn of the Nucleoprotein
Filament

- 5 To determine the minimum homology recognized by
recA in a synaptic complex, a series of
oligonucleotides 20 bases long was used that contained
varying amounts of homology at the 5' or 3' end to a
region of pBR322 flanking a Cla I site (see Table I).
- 10 This experiment not only establishes the minimum
homology recognized by recA in this assay, but also
definitively establishes whether the initiation of
pairing by recA exhibits directionality. The results
shown in Figure 15 indicate that recA can pair as few
- 15 as 8 bases of homology at either the 5' or 3' end
albeit at low efficiency (10% and 12%, respectively).
These results establish that the thresholds for
nucleoprotein filament formation and homologous pairing
are different and that either the 5' or 3' end of a
- 20 single-strand DNA can nucleate pairing. Fifteen bases
are required to form the structure that can carry out
the homology search, but only one-half of the bases in
this structure need be recognized and paired by recA.

Tabl 1. SEQUENCES OF OLIGONUCLEOTIDES CONTAINING
DECREASING AMOUNTS OF HOMOLOGY TO pBR322

20L Series

Sequence	Homology (bases)
5' TTGACAGCTTATCATCGATA 3'	20
GAATATATGCAT CATCGATA	10
GAATATATGCCAC ATCGATA	8
GAATATATGCCAT GTCGATA	6
GAATATATGCCAT GGAGATA	4
GAATATATGCCATGGATCGT	0

20R Series

Sequence	Homology (bases)
ATCATCGATA AAGCTTTAATG	20
ATCATCGAT AGAATATATGC	10
ATCATCGAG CGAATATATGC	8
ATCATCTCG CGAATATATGC	6
ATCAGATCG CGAATATATGC	4
CGACGATCGCGAATATATGC	0

Sequences in bold correspond to the bases homologous to pBR322.

Underlined sequences correspond to the position of the Cla I restriction site on the duplex.

EXAMPLE 12

Targeting of an Oligonucleotide by recA

A study was undertaken to determine to what extent recA can discriminate among several similar but distinct target sequences that reside within a single duplex molecule. Supercoiled M13mp18 replicative form DNA which has three Nde I recognition sites was incubated in the presence of recA with a 30-base oligonucleotide containing the Nde I recognition sequence as well as adjacent sequence from one of the three Nde I sites in M13mp18 (site I, see Figure 16A). The formation of a synaptic complex exclusively at site I was monitored by the appearance of a 6170 bp M13mp18 fragment following digestion of synaptic complexes with Nde I endonuclease. Such a fragment can only come about by cleavage at both sites II and III without cleavage at site I.

RecA was able to target pairing exclusively to site I in the majority of the DNA molecules (Figure 16B, lane 5). Such targeting required the presence of both oligonucleotide and recA (lanes 3 and 4). The presence of a 1080 bp fragment in all samples incubated with Nde I is a control for the extent of Nde I cleavage at both unprotected sites II and III. Quantitation of the amounts of each species in lane 5 indicates that the level of discrimination of recA for pairing at the target site I over sites II and III is about 7-8 fold under these conditions.

*

Experimental details relating to Examples 13-15.
Oligonucleotides:

Oligonucleotides were purified on an FPLC Mono Q column (Pharmacia) using a NaCl gradient from 100 mM to 1 M in 20 mM NaOH. Aliquots of peak fractions were

labeled with ^{32}P and run on polyacrylamid gels. The purest fractions were pooled, thanol precipitated, and dissolved in water. Concentrations were d termined by assuming that 1 OD unit at 260 nM is 33 μg .

5 RecA:

RecA protein was purified using a strain and a detailed protocol generously provided by Stephen Kowalczykowski of the Northwestern University Medical School in Chicago. The strain used was JC12772 (Uhlin
10 et al, *J. Bacteriol.* 148, 386 (1981)). The purification was based on the spermidine precipitation method (J. Griffith et al, *Biochemistry* 24, 158 (1985)), and employed a single-stranded DNA agarose column with ATP elution (Cox et al *J. Biol. Chem.* 256, 4676 (1981)) and a
15 Mono Q column to greatly reduce trace nuclease contamination. Removal of such contamination is important in order to avoid undesirable non-specific nicking of the DNA. The concentration of recA protein was measured using the extinction coefficient of $^{12}\text{E}_{280} =$
20 5.9 (Craig et al, *J. Biol. Chem.* 256, 8039 (1981)).

EXAMPLE 13

Sequence Specific Cleavage of Lambda DNA

A demonstration of the sequence-specific cleavage of lambda DNA at a single site is shown in Figure 18.
25 Lambda DNA is 48.5 kb in length, and contains 5 Eco RI sites (Daniels et al, in *Lambda II*, Hendrix et al, Eds. (Cold Spring Harbor, N.Y. 1983), pp. 519-678). The site located at nucleotide position 31,747 was selected for cleavage in order to cut lambda into two fragments
30 of 31.7 and 16.8 kb. An oligonucleotide 30 bases long and homologous to this position was synthesized, and Figure 18B shows the results of the cleavage using this oligonucleotide. Lane 1 shows uncut lambda DNA, and lane 2 shows a complete cleavage experiment.

Densitometry of lane 2 showed that 79% of the DNA was cleaved into the desired two fragments, and 19% of the DNA was uncut. A total of 2% of the DNA was cut at one of the four other Eco RI sites present in lambda DNA, caused by either nonspecific methylation protection by the recA protein and oligonucleotide complex, or incomplete methylation. Controls in lanes 3, 4, and 5 show the result of omitting recA protein, oligonucleotide, or methylase, respectively. Notably, in lane 3, omitting the recA protein resulted in incomplete methylation, possibly due to inhibition of the methylase by free oligonucleotide not coated with recA protein. Lane 4 DNA also showed slightly incomplete methylation, possibly because some nonspecific protection occurred from free recA protein binding to the duplex lambda DNA. This effect was seen more dramatically in Figures 19 and 20 where an oligonucleotide titration was done.

EXAMPLE 14

Sequence Specific Cleavage of E. coli DNA

Experimental details:

Wild type E. coli strain W3110 was obtained from the American Type Culture Collection and was grown overnight in Luria-Bertani medium to an optical density (OD) at 600 nm of 5. 5 ml of cells were pelleted (30 mg wet weight), washed once with 10 mM Tris-HCl (pH 7.2), 20 mM NaCl, and 100 mM EDTA, and resuspended in 1 ml of this buffer. The suspension was brought to 65°C, and added to 1 ml of 1.6% low melting point agarose (InCert agarose, FMC Bioproducts) and 4 ml of paraffin oil at 65°C. Microbeads 25 to 100 µm in diameter were formed by vortexing the suspension as described (M. McClelland, *Methods Enzymol.* 155, 22 (1987)). Beads were digested with lysozyme and proteinase K using the ImBed

kit (New England Biolabs) following the manufacturer's directions. Other lysozyme and proteinase K preparations gave equally good results. Beads were stored at 4°C. and were incubated at 50 mM EDTA for 30 minutes and equilibrated in 25 mM Tris-acetate (pH 7.5), 4 mM Mg-acetate, 0.4 mM dithiothreitol, and 0.5 mM spermidine immediately prior to use.

Results:

Application of the cleavage reaction to E. coli DNA is shown in Figure 19. In this case, a pair of oligonucleotides was added to obtain a fragment by cleavage at two sites. A large fragment was generated to test the power of the method. As shown in Figure 19A, one oligonucleotide was homologous to the uvrB gene, and the other to the topA gene. The oligonucleotides spanned Eco RI sites in each of these genes. The two genes are located 520 kb apart on the chromosome (Rudd et al, *Nucl. Acids Res.* 18, 313 (1990)), and at least 67 Eco RI sites are between these two target sequences (Kohara et al, *Cell* 50, 495 (1987)). Figure 19B shows the expected 520 kb band. A fairly sharp optimum was observed for oligonucleotide concentration of 5 nucleotide residues per recA protein monomer (lane 2). This was more clearly seen in the Southern blot in Figure 19'. Densitometry of the blot gave a yield of the fragment of 40%. As in the cleavage of lambda DNA, there was some non-specific protection from methylation by recA protein at lower oligonucleotide concentrations, and the 520 kb fragment was cleaved into smaller fragments. At higher oligonucleotide concentrations, the 520 kb fragment was also cleaved into smaller fragments, as would be expected from the result with lambda DNA in lane 3 of Figure 18B. An identical pattern with an optimum of 5

nucleotide residues per recA protein monomer was seen when the length of the oligonucleotides was increased from 30 to 60 bases, only in this case the yield of the 20 kb fragment increased to 60%. The 40 and 60% yields for the different pairs of oligonucleotides correspond to minimum single-side cutting efficiencies of 63 and 77%, respectively. This is close to the cutting efficiency on lambda DNA of 79%.

In certain applications, sequence information on both sides of an Eco RI site might be difficult to obtain. The fragment yield was therefore measured when the Eco RI recognition sequence, GAATTC, was at the 5' or the 3' end of a pair of oligonucleotides, instead of in the middle as in the previous study. When the recognition sequence was at the 5' end of the oligonucleotides (30 bases in length), the yield dropped two- to fourfold. When the sequence was at the 3' end, the yield dropped an additional twofold.

EXAMPLE 15

Sequence Specific Cleavage of Human DNA
Experimental Details:

Beads containing HeLa cell DNA were prepared by washing 1×10^8 cells (150mg wet weight) twice with phosphate buffered isotonic saline and processed as in Example 9 for the *E. coli* beads, except that the lysozyme digestion step was omitted.

Results:

The cleavage reaction was performed on human DNA with similar success. As the cystic fibrosis (CF) locus has been extensively mapped and sequenced (Rommons et al, *Science* 245, 1059 (1989); Riordan et al, *Science* 245, 1066 (1989); Zielenski et al, *Genomics* 10, 214 (1991)), it was used as a locus to test the method. Figure 20A is a simple schematic of the CF locus. An

Eco RI site is present in intron 1, and is 180 kb away from another Eco RI site in exon 19. At least 41 other Eco RI sites are found on this 180 kb stretch of genomic DNA (Rommons et al, *Science* 245, 1059 (1989)).

5 A gel stained with ethidium bromide is shown in Figure 20B, and shows how the production of smaller fragments occurred when the oligonucleotide concentration was lowered. This pattern was very reproducible and could be used as a guide to find the optimal concentration of
10 oligonucleotide without doing Southern blotting. The Southern blot of the gel is shown in Figure 20C. The greatest yield of the fragment was found in lane 3 (86%). A smaller yield (32%) was found in lane 2, but the background cleavage in lane 2 was much lower than
15 in lane 3. Thus, DNA from the 180 kb region of lane 2 probably was the most enriched in DNA from the CF locus. A control shown in lane S is the 270 kb fragment produced by digestion with Sfi I (Rommons et al, *Science* 245, 1059 (1989)). A predicted
20 48 kb fragment could also be produced by specific cleavage at exons 13 and 19.

It was also noted in Figure 20C that in lanes 3-6, the 180 kb fragment was further broken down to smaller fragments. These fragments were probably generated by
25 one specific cleavage at intron 1 or exon 19, and one nonspecific cleavage of the fragment internally. As the probe used is 50 kb from the exon 19 site, no fragments under 50 kb in length hybridized, although presumably they were present.

30

* * * *

The entire contents of all references cited above are incorporated herein by reference.

Certain aspects of the present invention have been described in some detail for purposes of clarity and

understanding. One skilled in the art will appreciate, however, that various changes can be made in form and detail without departing from the true scope of the invention.

WHAT IS CLAIMED IS:

1. A method of forming a three-stranded DNA molecule wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule, comprising

contacting a recombination protein with a double-stranded DNA molecule and with a single-stranded DNA molecule sufficiently complementary to one strand of said double-stranded DNA molecule to hybridize therewith, which contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that said three stranded DNA molecule is formed.

2. The method according to claim 1 wherein said recombination protein is *E. coli* recA or bacterial, or bacteriophage, recA-like protein.

3. The method according to claim 1 wherein said recombination protein is a eucaryotic recombinase or recA-like protein.

4. The method according to claim 3 wherein said recombinase protein is human recombinase protein.

5. A method of effecting cleavage of a double-stranded DNA molecule at a specific site comprising the steps of:

i) contacting a recombination protein with said double-stranded DNA molecule and with a single stranded DNA molecule sufficiently complementary to a portion of said double-stranded DNA molecule to hybridize therewith, wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed,

wh rein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule,

wherein one end of said single-stranded DNA molecule has attached thereto a cleavage moiety, which end is adjacent to said specific cleavage site; and

ii) exposing said cleavage moiety to conditions such that said cleavage moiety effects cleavage at said specific cleavage site.

6. The method according to claim 5 wherein said recombination protein is *E. coli* recA or bacterial, or bacteriophage, recA-like protein.

7. The method according to claim 5 wherein said recombination protein is a eucaryotic recombinase or recA-like protein.

8. The method according to claim 5 wherein said cleavage moiety is a chelating agent.

9. The method according to claim 5 wherein said cleavage moiety is a light-activated dye.

10. The method according to claim 5 wherein said cleavage moiety is attached to said single-stranded DNA molecule via a spacer molecule.

11. The method according to claim 10 wherein said spacer molecule comprises at least one methylene group.

12. The method according to claim 5 wherein said specific site is a target site for gene therapy.

13. The method according to claim 12 wherein said specific site is a target for gene inactivation or RNA inactivation.

14. A method of identifying the presence of a specific DNA sequence in a double-stranded DNA molecule comprising contacting a recombination protein with said double-stranded DNA molecule and with a single-stranded DNA molecule, which single-stranded DNA molecule is

sufficiently complementary to said specific sequence presented in said double-stranded DNA molecule, or the complement thereof, to hybridize therewith, wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed,

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule, and

wherein said single-stranded DNA molecule has a detectable label bound thereto;

ii) separating said three-stranded DNA molecule from unhybridized single-stranded DNA molecules; and

iii) detecting the presence of label associated with said three-stranded DNA molecule.

15. A method of protecting a double-stranded DNA molecule containing at least one restriction endonuclease recognition site from cleavage by said restriction enzyme comprising contacting a recombination protein with the double-stranded DNA molecule and with a single-stranded DNA molecule, which single-stranded DNA molecule is sufficiently complementary to a portion of one strand of said double-stranded DNA molecule that includes at least the at least one restriction endonuclease recognition site to hybridize therewith,

wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed, and

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule.

16. A method of inhibiting transcription of a specific gene sequence present on one strand of a double-stranded DNA molecule comprising contacting a recombination protein with said double-stranded DNA molecule and with a single-stranded DNA molecule, which single-stranded DNA molecule is sufficiently complementary to said gene sequence to hybridize therewith,

wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said gene sequence so that a three-stranded DNA molecule is formed, and

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule.

17. A method of selecting for a specific double-stranded DNA molecule present in a sample comprising:

i) contacting a recombination protein with said double-stranded DNA molecule and with a single-stranded DNA molecule, which single-stranded DNA molecule is sufficiently complementary to a specific sequence present in said double-stranded DNA molecule to hybridize therewith, wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed,

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule, and

wherein said single-stranded DNA molecule has a first member of a binding pair bound thereto;

ii) separating said three-stranded DNA molecule from unhybridized single-stranded DNA molecules;

iii) contacting said three-stranded DNA molecule with a second member of said binding pair; and

iv) isolating said three-stranded DNA molecule bound to said second member of said binding pair.

18. A method of effecting cleavage of a double-stranded DNA molecule containing at least two restriction endonuclease recognition sites from cleavage at a first of said sites by a restriction enzyme specific for said sites comprising:

i) contacting a recombination protein with the double-stranded DNA molecule and with a single-stranded DNA molecule, which single-stranded DNA molecule is sufficiently complementary to a portion of one strand of said double-stranded DNA molecule that includes the first of said restriction endonuclease recognition sites to hybridize therewith,

wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed, and

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule, and

ii) modifying the at least one other of said sites so as to render it protected from said restriction enzyme;

iii) dissociating said single-stranded DNA molecule from said double stranded molecule; and

iv) cleaving said double-stranded molecule at said first of said restriction sites.

19. A method of protecting a sequence of a double-stranded DNA molecule from modification by a modifying agent comprising contacting a recombination protein with the double-stranded DNA molecule and with a

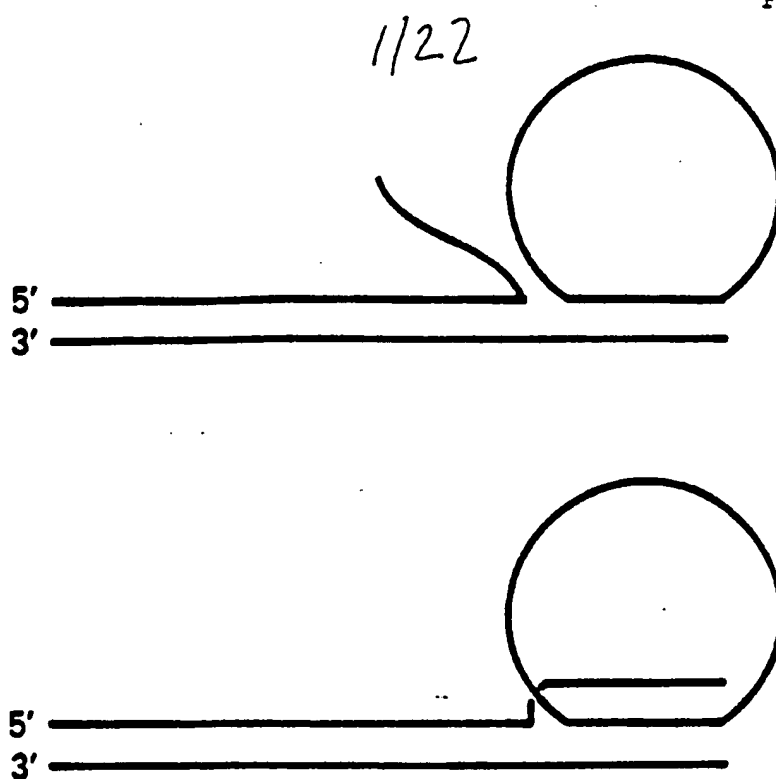
single-stranded DNA molecule, which single-stranded DNA molecule is sufficiently complementary said sequence of said double-stranded DNA molecule to hybridize therewith,

wherein said contacting is effected under conditions such that said single-stranded DNA molecule hybridizes to said double-stranded molecule so that a three-stranded DNA molecule is formed, and

wherein each strand of said three-stranded DNA molecule is hybridized to at least one other strand of said three-stranded DNA molecule.

20. The method according to claim 19 wherein said modification is methylation and said agent is a methylase.

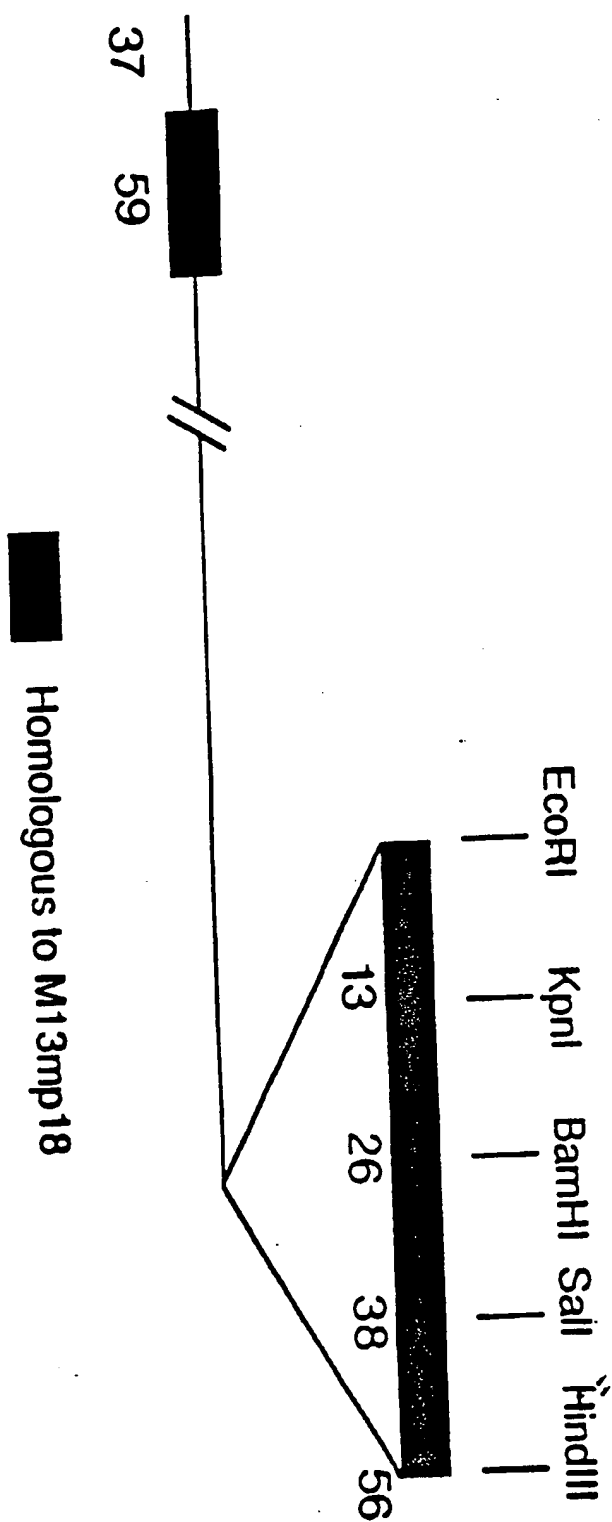
FIGURE 1

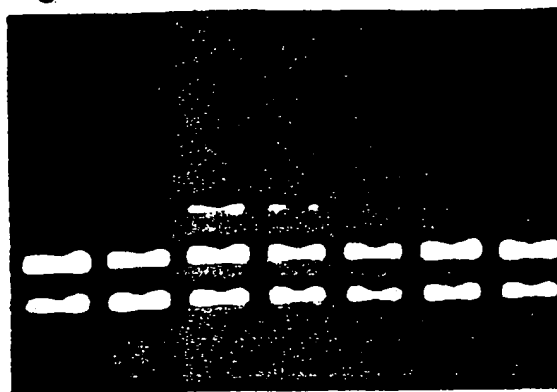
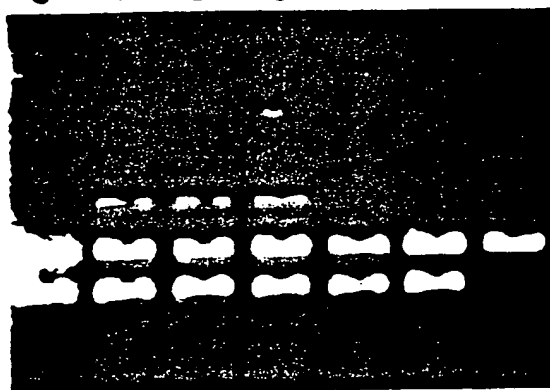
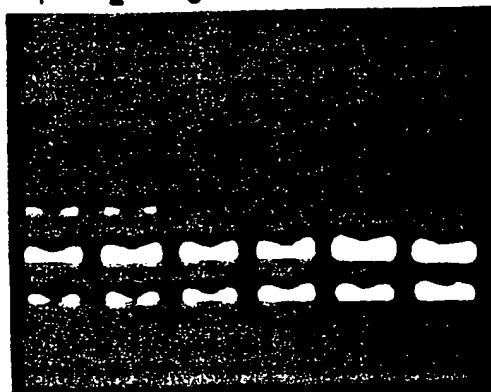


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A

pGem 4



BHomology
LaneC 56 38 26 13 5 5.
1 2 3 4 5 6joint
molecule →
ds →
ss →**C**Homology
LaneC 56 38 26 13 5
1 2 3 4 5 6joint
molecule →
ds →
ss →**D**Homology
Lane56 38 26 13 5 5
1 2 3 4 5 6joint
molecule →
ds →
ss →

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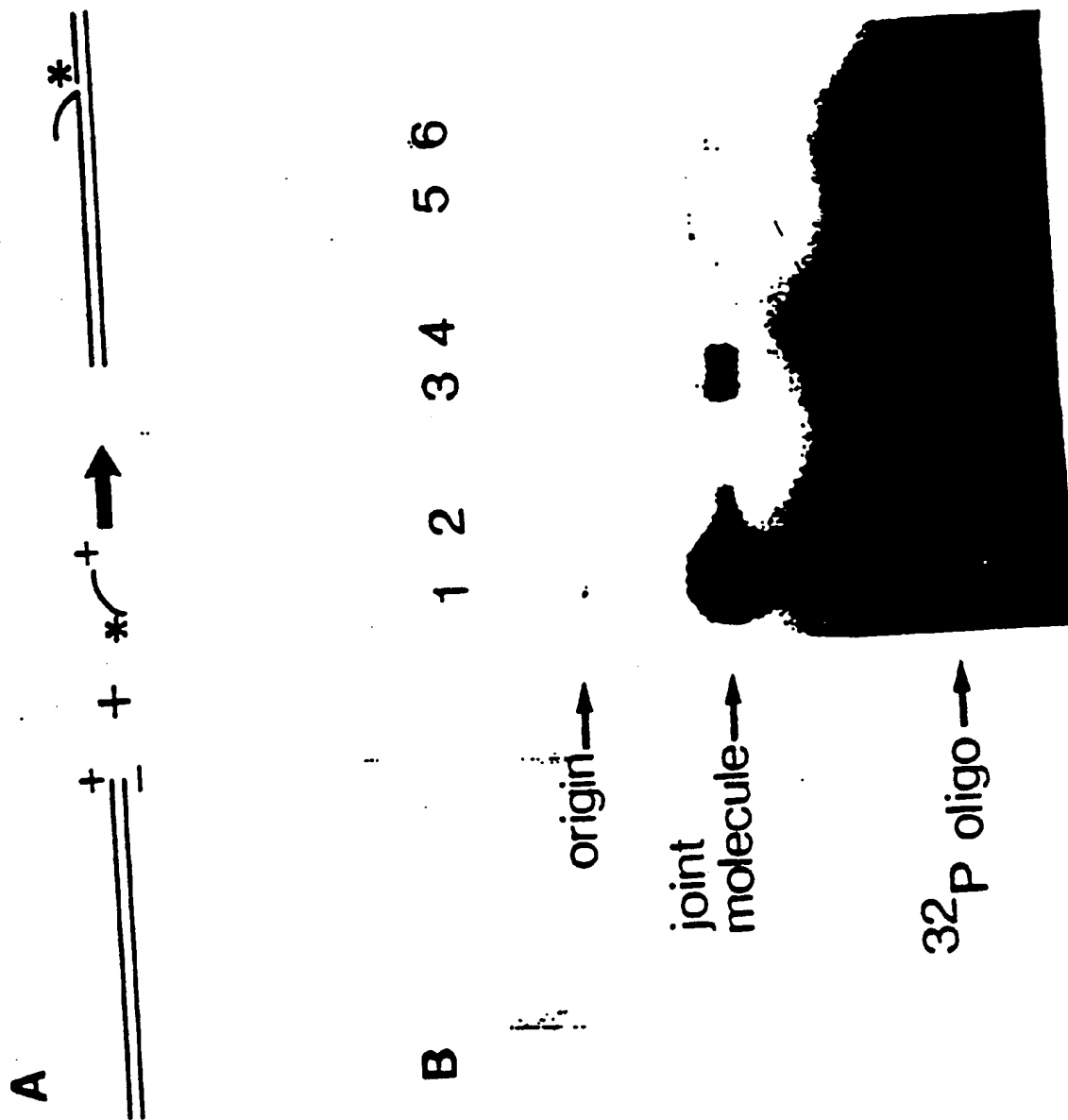


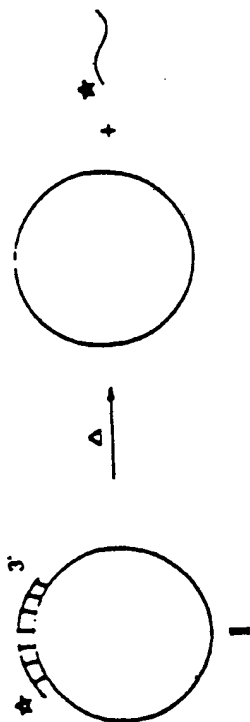
FIGURE 3

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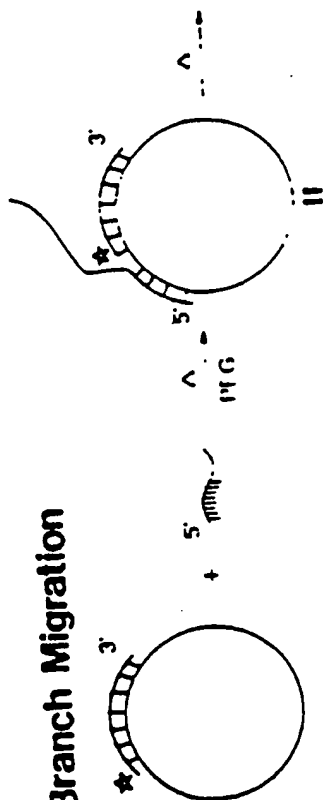
FIGURE 4

A

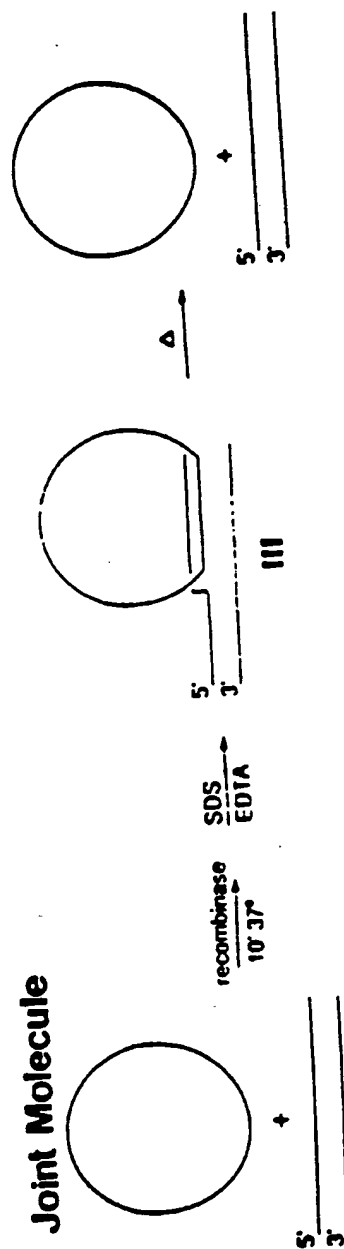
Duplex



Branch Migration



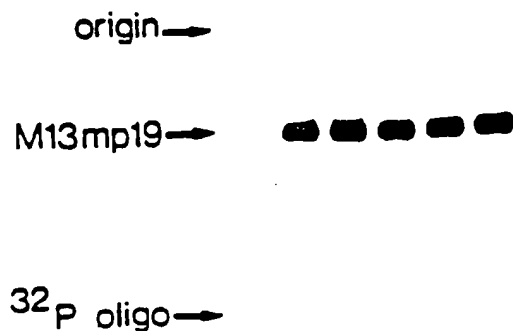
Joint Molecule



B

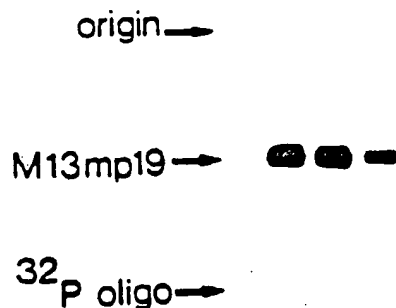
I. Duplex

0° 37° 45° 55° 65° 75° 85°

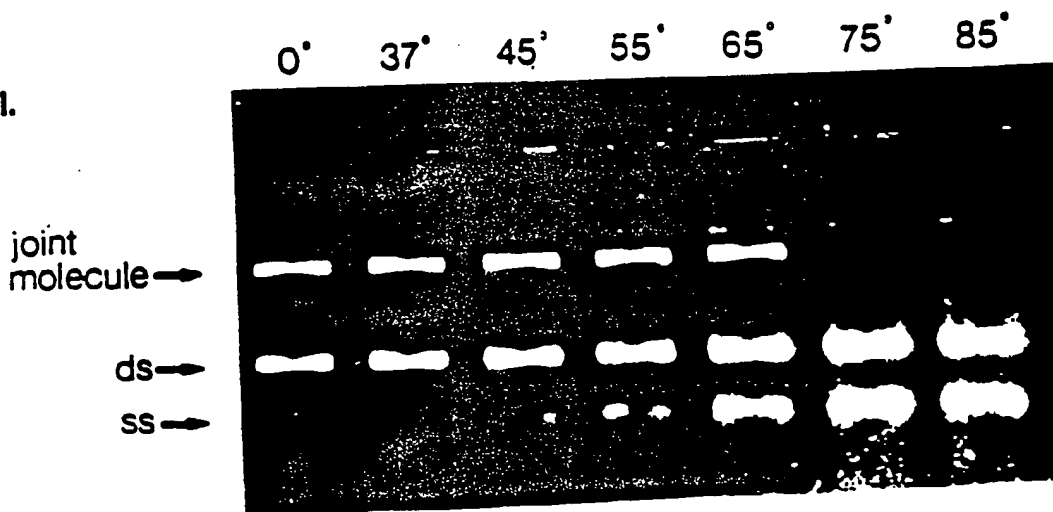


0° 37° 45° 55° 65° 75° 85°

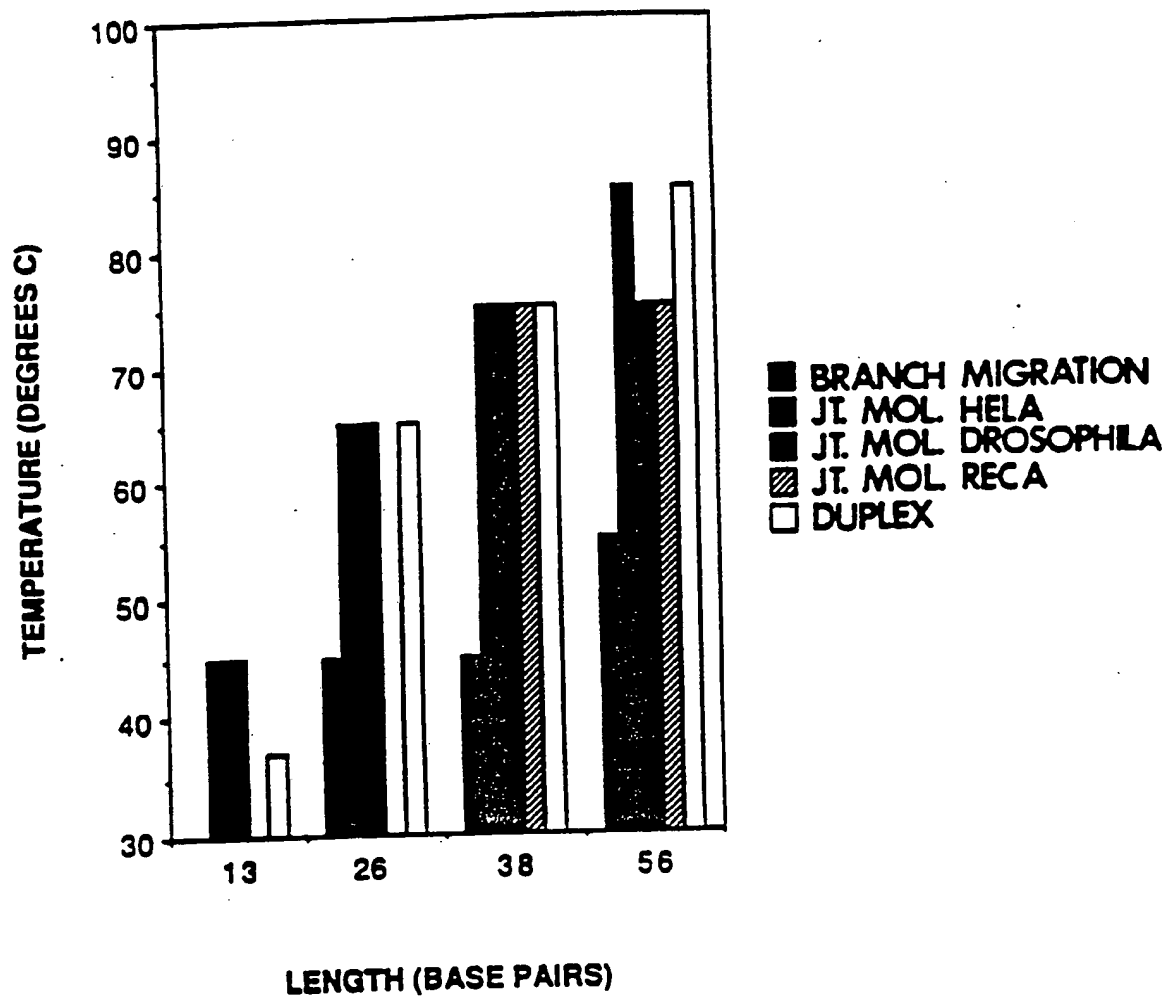
II. Branch Migration



III.

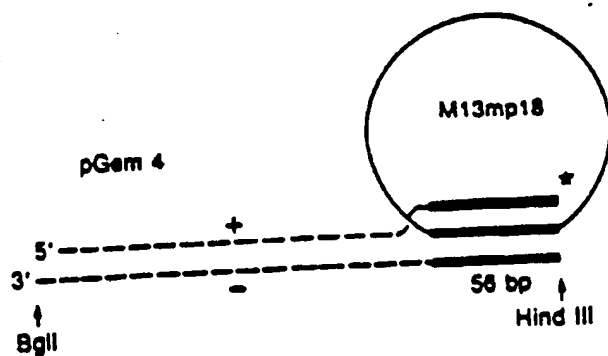


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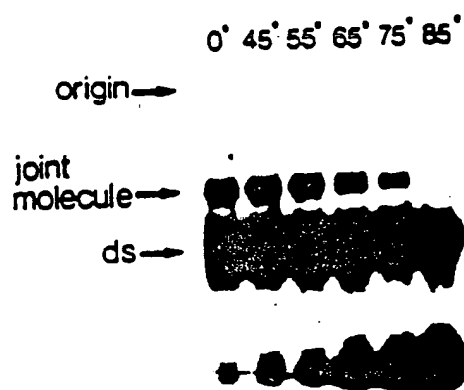


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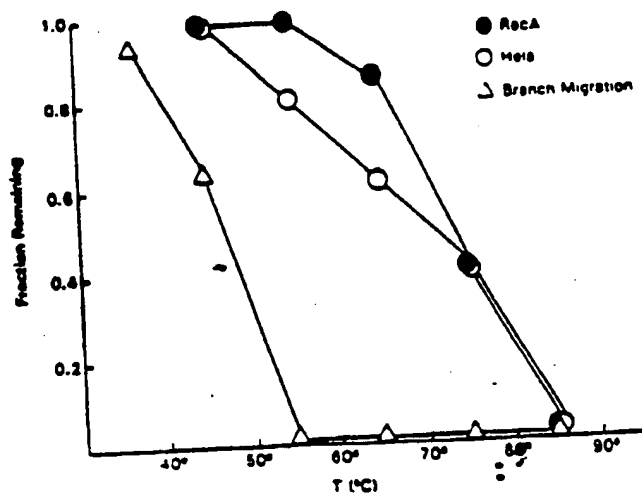
A



B

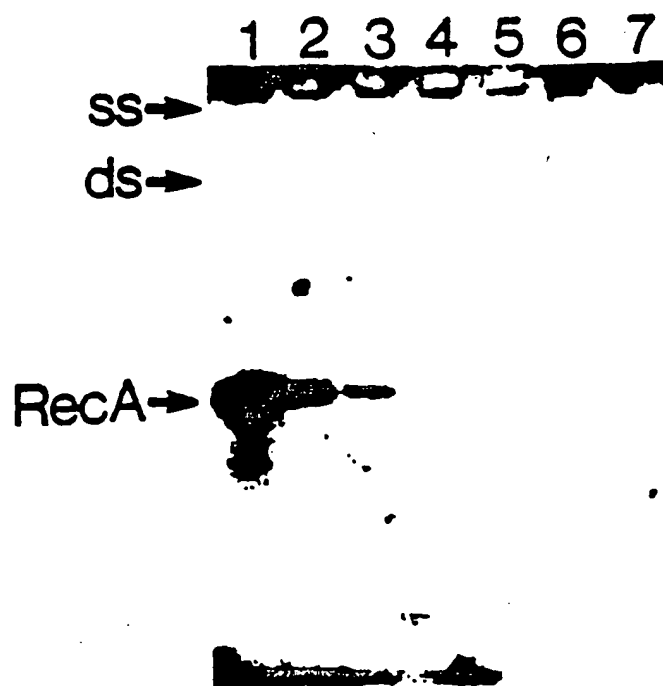


C

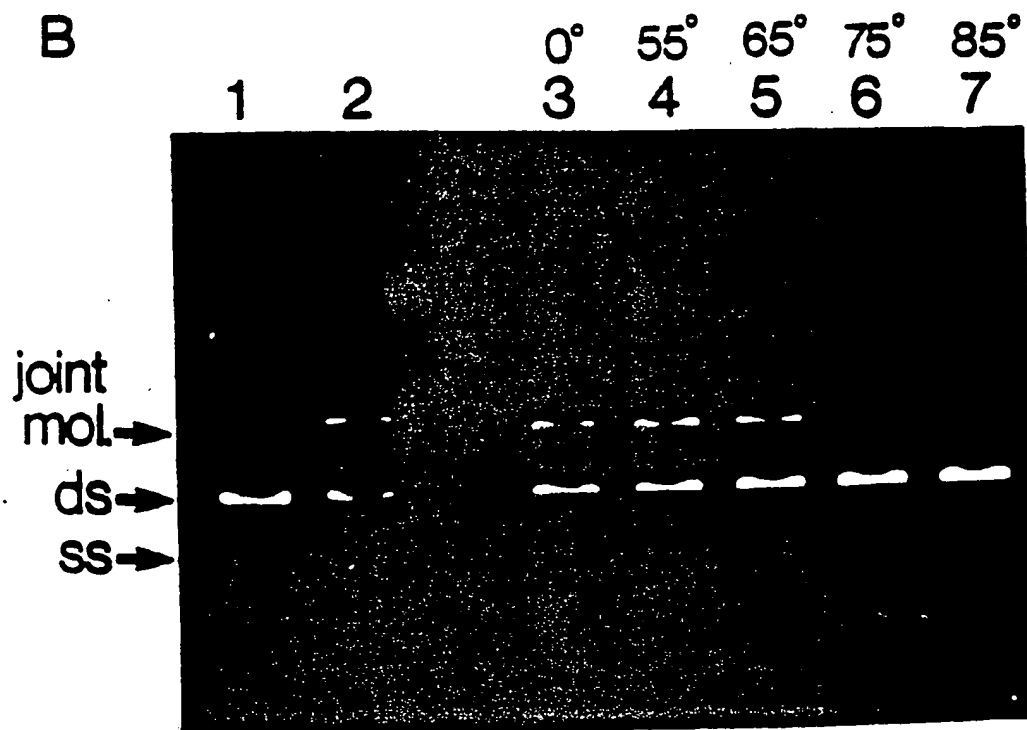


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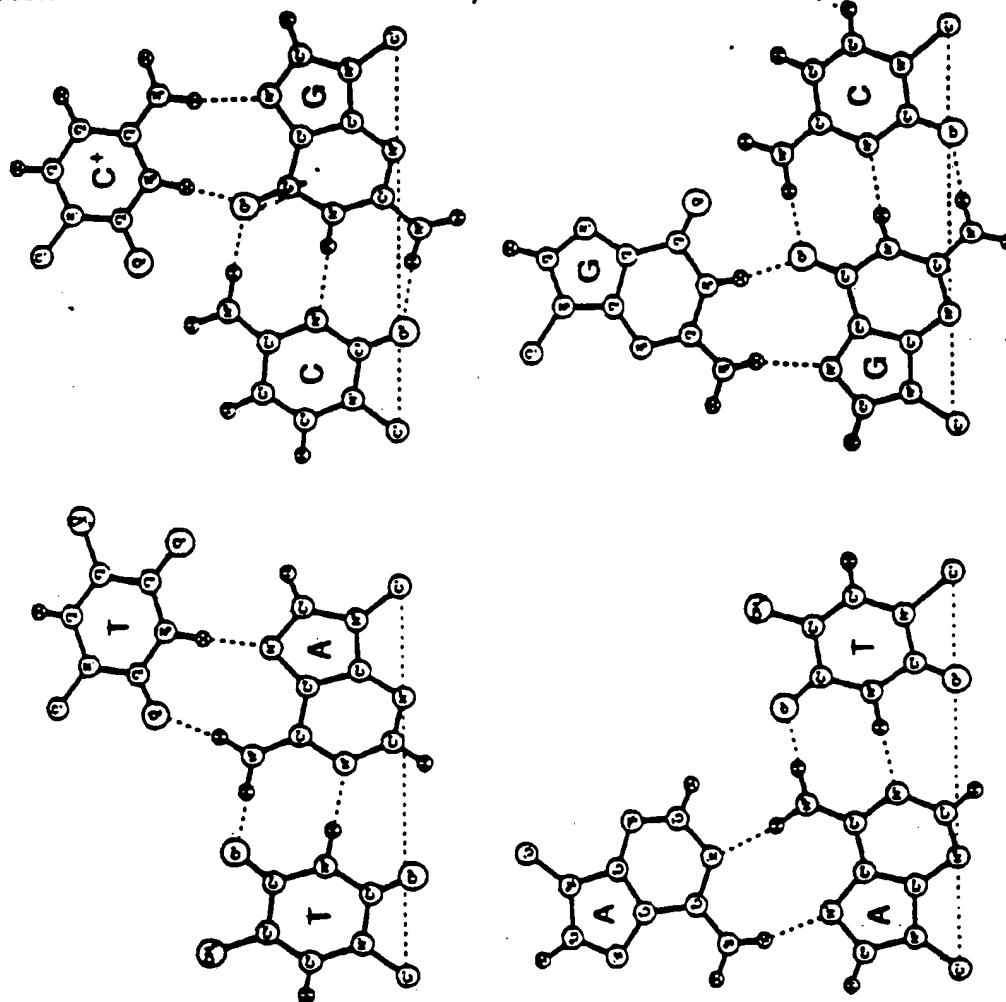
A



B



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B

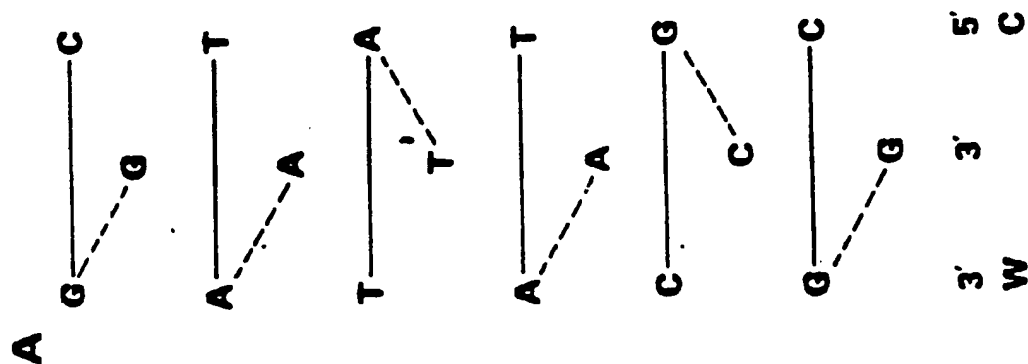
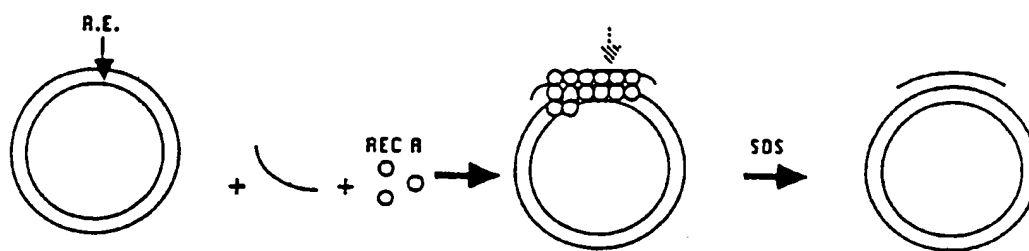


FIGURE 8

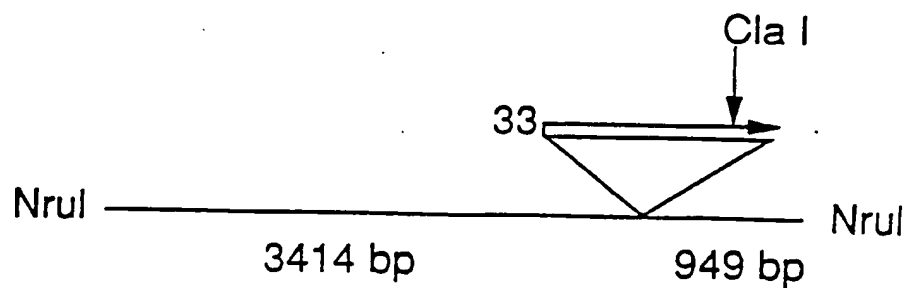
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FIGURE 9



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FIGURE 11

A

pBR322

B

LANES	1	2	3	4	5	6
OLIGO	-	H	N	H	N	-
REC A	-	-	-	+	+	+
Cla I	-	+	+	+	+	+

4363 bp →
3413 bp →

949 bp →

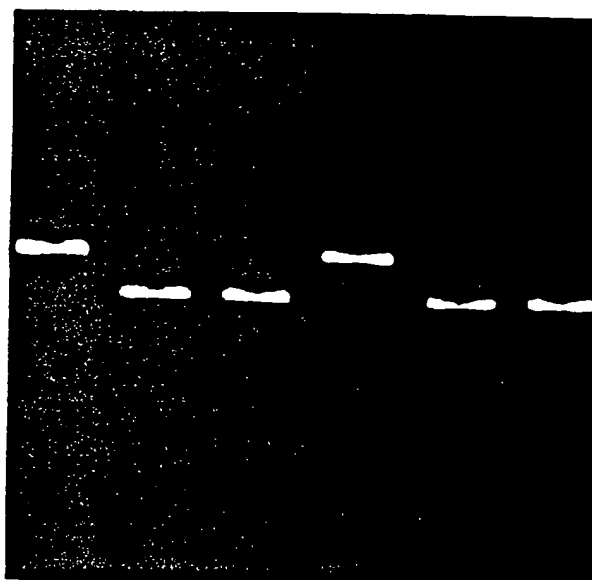
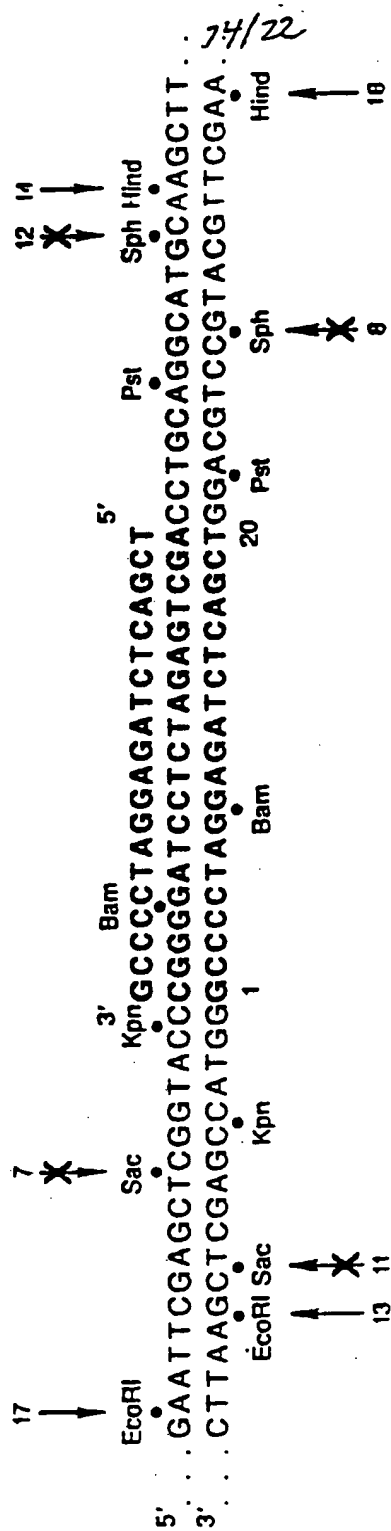


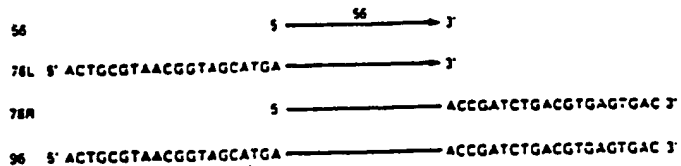
FIGURE 12



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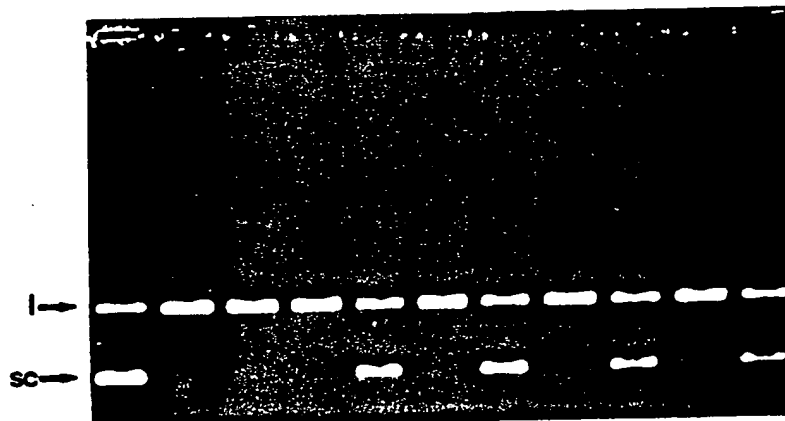
FIGURE 13

A



B

	1	2	3	4	5	6	7	8	9	10	11
oligo	-	-	-	-56-	-	-76L-	-	-76R-	-	-96-	-
RecA	-	-	+	-	+	-	+	-	+	-	+
BamHI	-	+	+	+	+	+	+	+	+	+	+



C

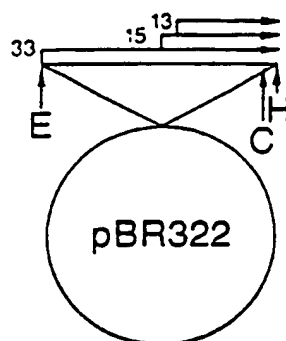
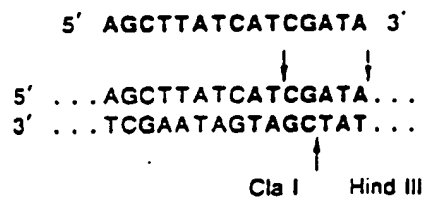
	1	2	3	4	5	6	7	8
oligo	56	56	76L	76L	76R	76R	96	96
RecA	-	+	-	+	-	+	-	+

SC →



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FIGURE 14

A**B****C**

LANES	1	2	3	4	5	6	7	8	9	10
OLIGO 15b.	-	+	-	+	+	-	+	+	-	-
REC A	-	-	+	+	-	+	+	-	+	-
REST. ENZ.	-	Hind III			Cla I			Eco RI		

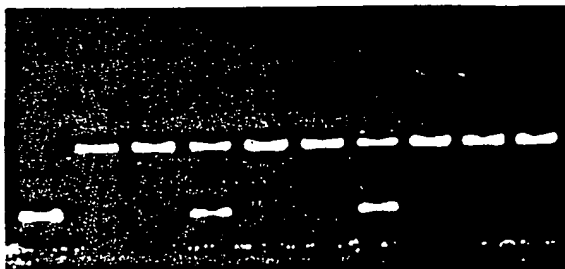
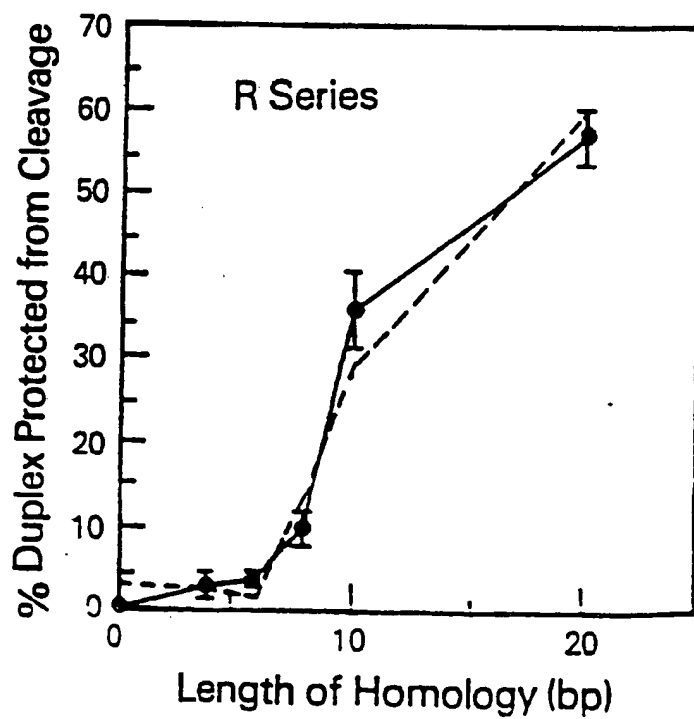
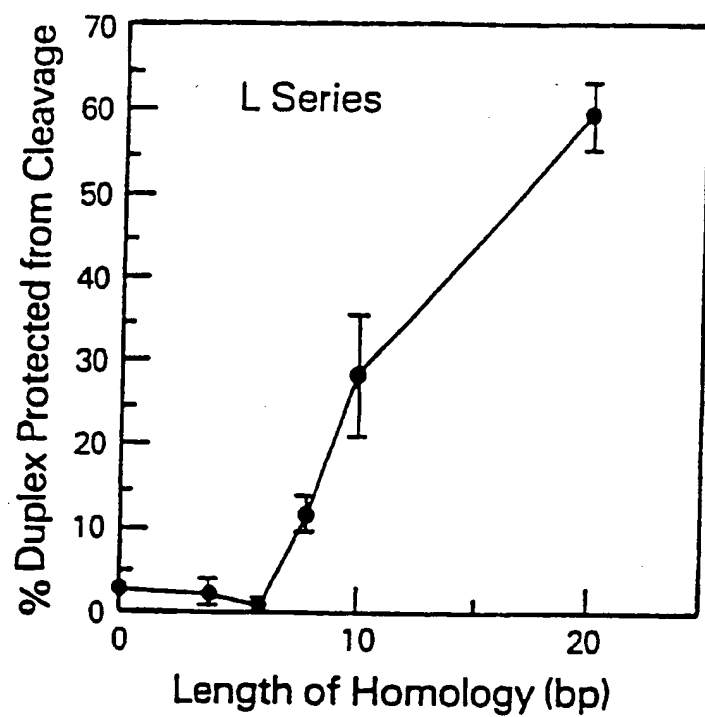
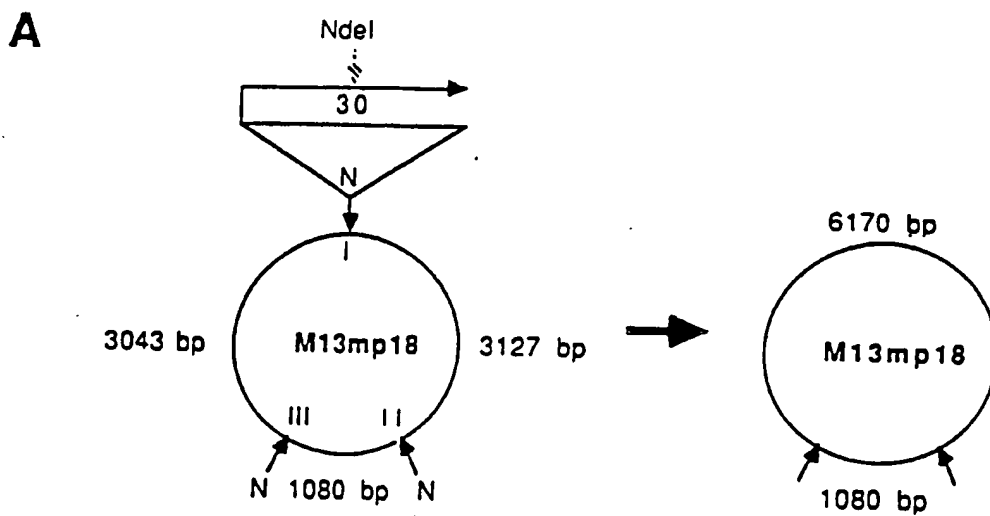


FIGURE 15

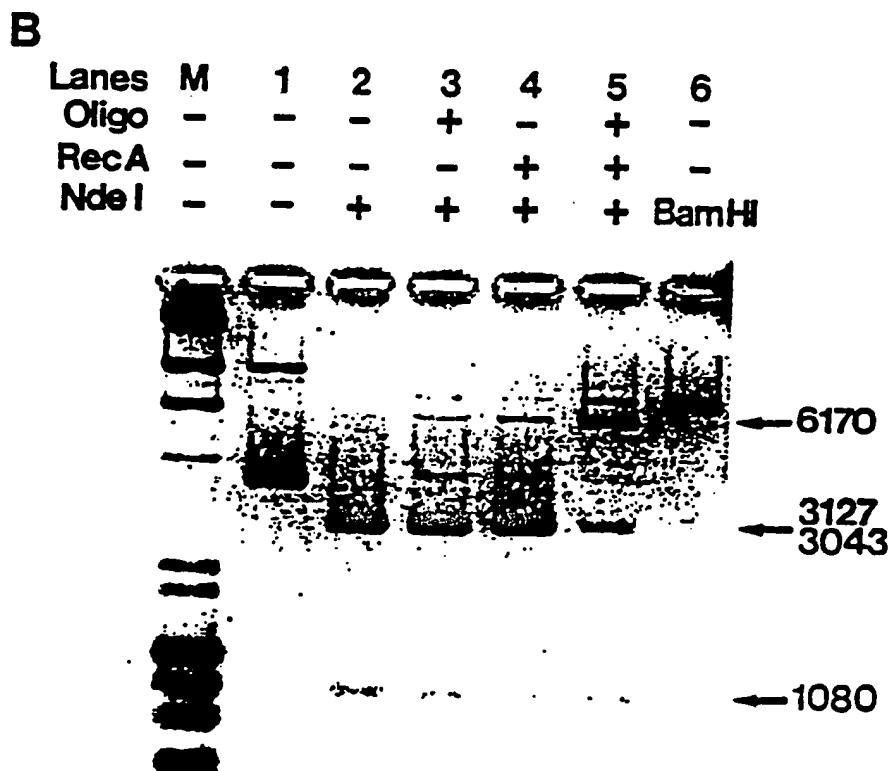


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FIGURE 16



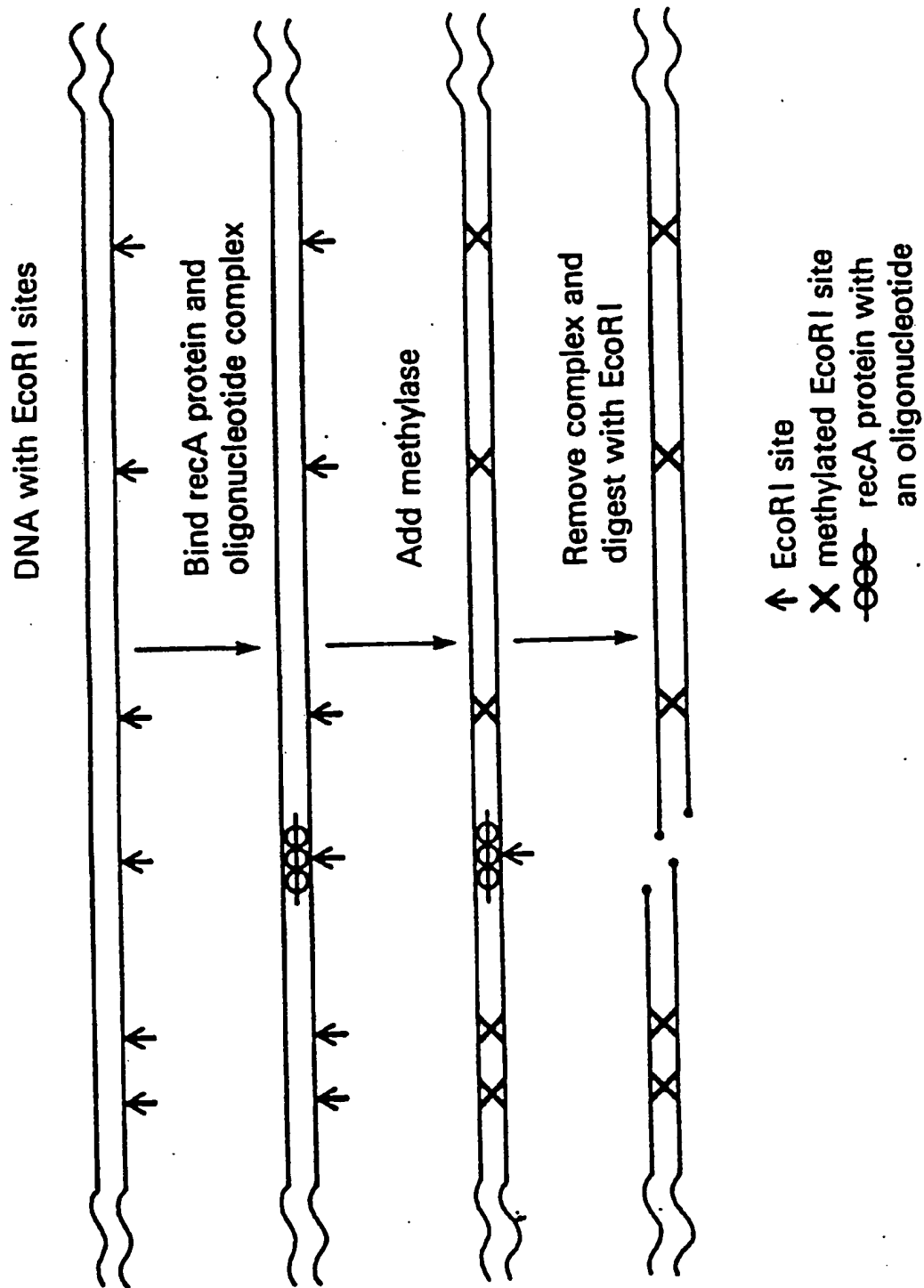
M13 mp18 has three cleavage sites for NdeI (N)



1211111 Y LUMEN1111111 1111111

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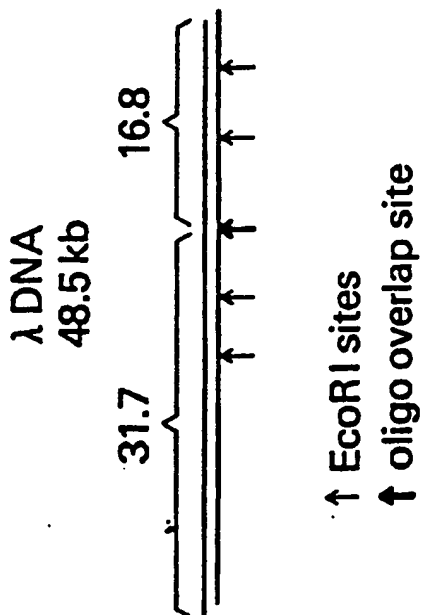
FIGURE 17



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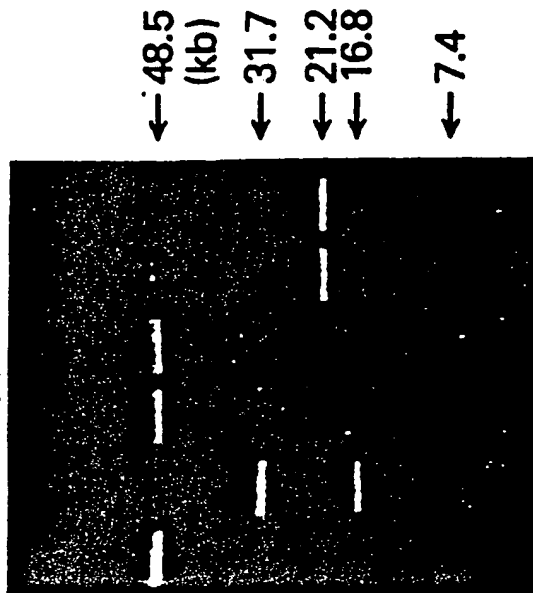
FIGURE 18

A



B

Lanes	1	2	3	4	5	6
RecA	-	+	-	+	+	-
Oligo	-	+	+	-	+	-
Methylase	-	+	+	+	-	-
EcoRI	-	+	+	+	+	+



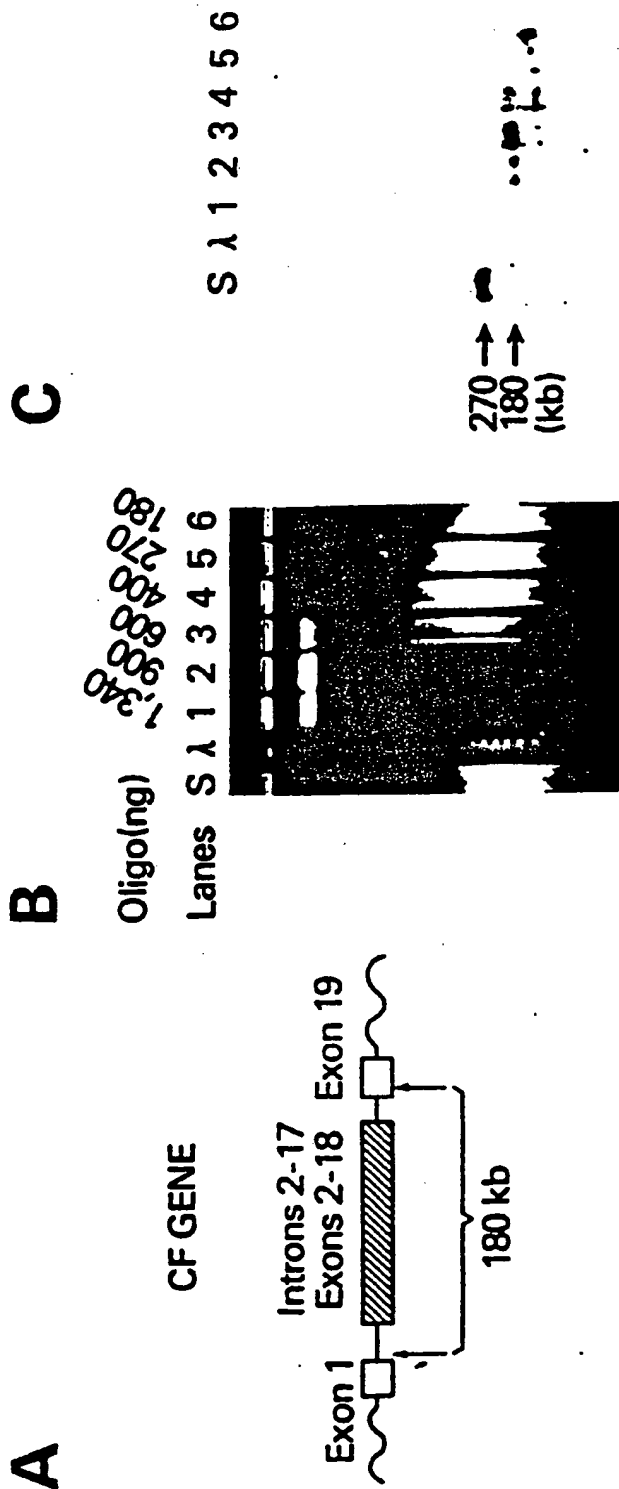
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FIGURE 19



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FIGURE 20



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08200

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5) : C12N 15/00; C12P 19/34; C12 Q 1/68 US CL : 435/6, 91, 172.3						
II. FIELDS SEARCHED <div style="text-align: center;">Minimum Documentation Searched⁴</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 20%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">U.S.</td> <td style="padding: 5px;">435/6, 91, 172.3, 196, 199; 935/16, 78, 80</td> </tr> </table> <div style="text-align: center;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched⁵</div>			Classification System	Classification Symbols	U.S.	435/6, 91, 172.3, 196, 199; 935/16, 78, 80
Classification System	Classification Symbols					
U.S.	435/6, 91, 172.3, 196, 199; 935/16, 78, 80					
CA-FILE: 1967-1991 BIOSIS: 1969-1991						
III. DOCUMENTS CONSIDERED TO BE RELEVANT¹⁴						
Category [*]	Citation of Document ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸				
X Y	US, A. 4,888,274 (RADDING ET AL.) 19 DECEMBER 1989. SEE ENTIRE DOCUMENT.	1-3, 14 5-13, 17-18				
X Y	JOURNAL OF MOLECULAR BIOLOGY, VOLUME 193, ISSUED 1987, S.C. KOWALCZYKOWSKI ET AL, "EFFECTS OF <u>ESCHERICHIA COLI</u> SSB PROTEIN ON THE SINGLE-STRANDED DNA-DEPENDENT ATPASE ACTIVITY OF <u>ESCHERICHIA COLI</u> : RECA PROTEIN", PAGES 97-113. SEE ENTIRE DOCUMENT.	1-3 5-13, 17, 18				
X Y	L. STRYER "BIOCHEMISTRY", SECOND EDITION, PUBLISHED 1981 BY W.H. FREEMAN AND COMPANY (SAN FRANCISCO) PAGES 754-756. SEE ENTIRE DOCUMENT.	1-3 5-13, 17 AND 18				
X Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 264, NO.9, ISSUED 25 MARCH 1989, HSIEH ET AL, "FORMATION OF JOINT DNA MOLECULES BY TWO EUKARYOTIC STRAND EXCHANGE PROTEINS DOES NOT REQUIRE MELTING OF A DNA DUPLEX," PAGES 5089-5097. SEE ENTIRE DOCUMENT.	1-4, 14 5-13, 17 AND 18				
X	SCIENCE, VOLUME 245, ISSUED 18 AUGUST 1989, L.J. MAHER III ET AL, "INHIBITION OF DNA BINDING PROTEINS BY OLIGONUCLEOTIDE-DIRECTED TRIPLE HELIX FORMATION", PAGES 725-730. SEE ENTIRE DOCUMENT.	15, 16, 19 AND 20				
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>						
IV. CERTIFICATION						
Date of the Actual Completion of the International Search ² <div style="text-align: center;">22 JANUARY 1992</div>	Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em;">26 MAR 1992</div>					
International Searching Authority ¹ <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center;"> JAMES MARTINELL </div>					

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET		
X	BIOCHEMISTRY, VOLUME 28, ISSUED 1989, J-C FRANCOIS ET AL., "INHIBITION OF RESTRICTION ENDONUCLEASE CLEAVAGE VIA TRIPLE HELIX FORMATION BY HOMOPYRIMIDINE OLIGONUCLEOTIDES", PAGES 9617-9619. SEE ENTIRE DOCUMENT.	15, 16 AND 19
X	SCIENCE, VOLUME 241, ISSUED 22 JULY 1988, M. COONEY ET AL., "SITE-SPECIFIC OLIGONUCLEOTIDE BINDING REPRESSES TRANSCRIPTION OF THE HUMAN C-MYC GENE IN VITRO", PAGES 456-459. SEE ENTIRE DOCUMENT.	16 AND 19
V. <input type="checkbox"/> OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹ This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons: 1. <input type="checkbox"/> Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely: 2. <input type="checkbox"/> Claim numbers , because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically: 3. <input type="checkbox"/> Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).		
VI. <input type="checkbox"/> OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING² This International Searching Authority found multiple inventions in this international application as follows: 1. <input type="checkbox"/> As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. 2. <input type="checkbox"/> As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: 3. <input type="checkbox"/> No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 4. <input type="checkbox"/> As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee. Remark on protest: <input type="checkbox"/> The additional search fees were accompanied by applicant's protest. <input type="checkbox"/> No protest accompanied the payment of additional search fees.		

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, U.S.A., VOLUME 82, ISSUED FEBRUARY 1985, G.B. DREYER ET AL, "SEQUENCE-SPECIFIC CLEAVAGE OF SINGLE-STRANDED DNA: OLIGODEOXYNUCLEOTIDE-EDTA-F (II)", PAGES 968-972. SEE ENTIRE DOCUMENT.	5-13,17, AND 18
X Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 265, NO.28, ISSUED 05 OCTOBER 1990, UMLAUF ET AL, "TRIPLE-HELICAL DNA PAIRING INTERMEDIATES FORMED BY RecA PROTIEN", PAGES 16898-16912. SEE ENTIRE DOCUMENT.	<u>1-3</u> 5-13,17 AND 18

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